IMPORTANCE OF ASYMPTOMATIC MALARIA AND ITS INFECTIVITY TO *ANOPHELES* MOSQUITOES IN MAE HONG SON PROVINCE, THAILAND

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July 2002

ABSTRACT

This study aimed to record the prevalence of asymptomatic malaria and the development of malaria parasites in laboratory-bred *Anopheles minimus* mosquitoes fed on the blood of people with or without malaria symptoms. The intention was to identify where the main reservoir of infection was maintained. The study was conducted in three cantons in Muang district, Mae Hong Son Province, Northwest Thailand.

Chapter 3 describes studies of epidemiology of malaria in this area, based on two cross-sectional surveys of populations in villages and people who visited a malaria clinic. A questionnaire was conducted in the local languages by trained interviewers, to identify people suffering from fever.

The parasite prevalence was 0.7% of all the surveyed populations. The prevalence of slide positivity among males was significantly higher than females. All those with positive slides who had a body temperature >37.4°C reported fever. Some without measured body temperature >37.4°C also reported fever. Those people may have had fever a few hours previously. Contrary to expectation *P. falciparum* infections did not cause fever more than *P. vivax* infections. The prevalence of asymptomatic malaria was 47.1% among those who were slide positive which corresponded to about 0.5% of the interviewed population. From the Clinic, 101 patients with malaria infection were interviewed. More than half of the infections had high parasitaemia.

When people thought they had malaria, the malaria clinic and the hospital facilities were most commonly used, with the largest number going to the Clinic. A significantly higher malaria prevalence was found in the people who reported not having any form of mosquito protection. More than 99% stated that they owned mosquito nets, but only 44% of the nets had been treated with insecticide.

Chapter 4 reports a study of the infectiousness of malaria patients to *An. minimus*. Direct feedings were conducted on adults >15 years old and who gave their consent. All of these people had blood slides positive for *P. falciparum* or *P. vivax* and had been located from the village surveys (n = 28) or the Clinic (n = 92). After feeding, the mosquitoes were held for 7-9 days and then dissected and examined for oocysts. Feeding on about 40% of human subjects yielded some mosquitoes with oocysts. There was a significantly lower probability of infection with *P. falciparum* than *P. vivax*. Symptomatic or non-symptomatic people with parasites were equally able to infect mosquitoes. There was a significant association of probability of mosquito infection with presence of observable gametocytes, but there were some individuals without observable gametocytes who infected mosquitoes.

PCR on 58 subjects with negative slides showed that 30% were positive for *P. falciparum*, but none yielded oocysts after mosquito feeding. PCR was also used to identify the parasite species in the mosquito's gut. The results revealed that none of the donors with visible *P. falciparum* infections carried cryptic *P. vivax* infections which could yield *P.vivax* positivity in mosquitoes fed on them.

The occurrence of some cases with undetectable gametocytes who could infect mosquitoes suggests the question "Do gametocytes selectively enter the mosquito's proboscis during blood feeding"? However, a small study showed that the gametocyte density in the mosquito blood meals did not differ significant from that in the corresponding finger pricks, indicating that gametocytes do not selectively enter a mosquito's proboscis. The occurrence in some cases of high oocyst counts in mosquitoes fed on blood with no observable gametocytes remains unexplained.

From the results, on infection of mosquitoes by different categories of people an attempt was made to estimate the number of people in the catchment area of the Clinic who were reservoirs of infection. On the basis of the number of patients visiting the Clinic per day, it was concluded that the main reservoir of infection for mosquitoes was not in patients feeling ill enough to be motivated to come to the Clinic. The present study suggests that directing anti-gametocyte drugs to all feverish

patients in the villages could have a major impact on the reservoir of infection. However, in Thailand there have not yet been reports on the infectivity of the gametocytes in mosquitoes after treatment of patients with gametocytocidal drugs (e.g. primaquine or artesunate). Such studies are strongly recommended.

GLOSSARY OF TERMS AND ABBREVIATIONS

ABER	Annual Blood Examination Rate
ACD	Active Case Detection
API	Annual Parasite Incidence
DDT	Dichloro-diphenyl-trichloroethane
G6PD	Glucose-6-phosphate dehydrogenase
HC	Health Centre
IRC	Immigrant Rescue Commission
IRS	Indoor residual spraying
MBS	Mass Blood Survey
MH	Mantel-Haenszel
PCD	Passive Case Detection
SCD	Special Case Detection
SPR	Slide Positive Rate
VBDC	Vector-borne Disease Control Centres
VBDO	Office of Vector-borne Disease Control
VBDU	Vector-borne Disease Control Unit
VHW	Village Health Worker
VMV	Village Malaria Volunteer
WBC	White Blood Cell
WHO	World Health Organisation

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ACKNOWLEDGEMENTS

I am grateful to the Royal Thai Government which financially supported my Ph.D. at the London School of Hygiene and Tropical Medicine and to WHO/TDR for funding my field work. I also thank the Faculty of Medicine, Thammasat University for allowing me leave of absence for further study.

I wish to thank the chiefs and all the people of Pang Moo, Huai Pha and Mok Cham Pae cantons, without whose co-operation and support my work would not have been possible.

I give very special thanks and sincere appreciation to my supervisor, Professor Chris Curtis for his constant encouragement, advice, guidance and being patient during writing up the thesis. I should also not forget to thank my former supervisor, Dr Roger Webber for his encouragement and advice. I wish to thank Dr Ilona Carneiro for her advice on statistical analysis. I am also thankful to Dr Apinun Aramratana, for his suggestions on collecting and management of data and Dr Yapha Rongsriyam for her recommendation to work on mosquitoes.

I am grateful indebted to Dr Somsak Prajackwong, the Director of the Office of Vector-borne Disease Control No.2, Chiang Mai (VBDO), for providing me with a research team, vehicle, an office during the fieldwork and facilities for rearing mosquitoes. Special thanks go to Miss Wannapa Suwonkerd for her useful suggestions, moral support throughout the fieldwork and friendship when we were together in Chiang Mai. I would like to thank Mr Pongnarin Dee-in, Mr Somkid Sumonmard, Mrs Suparp Chatchatreechan and the staff in the Entomological Department of VBDO for their help in looking after and dissecting mosquitoes. I also thank the staff in the Investigation Department of VBDO for their kindness in re-checking and counting the parasite densities in blood films. Special thanks are extended to the staff of the Vector-borne Disease Control Centre No.21 (VBDC), especially Mrs Siripan Chopdee and Mr Amnoy Japanja. I am very grateful to my research team in Mae Hong Son Province, Mr Boontawee Corthong and his staff

from the Vector-borne Disease Control Unit No.8. This thesis would not have been possible without their support and hard work.

I wish to acknowledge the help and co-operation of Dr Georges Snounou and staff of the Unit de Parasitologie Biomedicale, Institut Pasteur, Paris, Drs Ali Alloueche and Jill Curtis from the London School of Hygiene and Tropical Medicine (LSHTM) for their support during the PCR study. I want to thank John Williams and his team for re-checking slides and the staff of the LSHTM insectary for providing mosquitoes for practice dissection. I also thank all staff of the DCVB unit, the librarian and all my friends at the LSHTM for their support.

Finally, I am especially indebted to Dr Walter Bob Taylor, my sisters, my brother and my parents for their love and understanding which gave me the courage to finish this thesis.

DEDICATION

To my parents

With love and gratitude

and

To everyone who donated blood by finger prick and mosquito feeding during the present study

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Climatic zonation and health service in Thailand

Thailand can be divided into three different topographical areas; plain areas in the central region of the country, highland areas mostly in the Northeast, and mountainous areas in the North and the Southeast.

There are three types of climate in Thailand as follows:

1. Tropical rain forest: this refers to the coastal areas in the East and the South, with heavy rainfall all year round and tropical rain forests.
2. Tropical monsoon climate: the Southwest and Southeast coasts with monsoons and a very high average annual rainfall.
3. Savannah climate: this area covers the Southwest (heavy rains), the Central region, the North and the Northeast with a monsoon season and a dry cool season.

This project was carried out in the North of the country (see more detail in section 1.10.1)

The health service system is a complex mixture of public and private providers but a major source of services is the Ministry of Public Health. The number of government hospitals is nearly 1,000. The number of health centres, which are established at the canton level, is about 9,600 (Malaria Division, 1999). In addition the number of private clinics is nearly 12,200 and private hospitals number about 500. Unfortunately, most of the private health services are located in urban areas. In 1997, life expectancy at birth was estimated as 70 and 75 years for men and women, respectively (Malaria Division, 1997).

The public provider which was involved in this project is the Vector-borne Disease Control Unit No.8 (VBDU), Mae Hong Son. This office is under the responsibility of the Office of Vector-borne Disease Control No.2 (VBDO), Chiang Mai, Department of Communicable Disease, Ministry of Public Health.

1.2 Malaria in Thailand

Malaria is still an important infectious disease in Thailand, despite decades of successful control programmes and dramatic reductions in morbidity and mortality. Malaria transmission in the forested areas is intense, due to the presence of highly efficient vectors, and significant population movement.

Five notable malaria epidemic events in Thailand are presented in Table 1.1.

Table 1.1 Summary of circumstances surrounding five important malaria epidemic events in Thailand in the last 20 years.

Year	Where	How/Why	Effect
1980-1984	Thai-Laos and Thai-Cambodian border	- Migration of people from Cambodia and Laos into Thailand because of serious fighting in their own countries. - *P. falciparum* was the dominant species.	Resistance to sulfadoxine-pyrimethamine (Fansidar).
1986-1988	Southern Thailand	- After increase in the price of coffee, people migrated into forest for coffee plantation including migrants from other parts of the country. Population of *An.minimus* s.l. increased. - *P. vivax* was the main species.	Malaria was transmitted into the local population and they refused house spraying.
1988-1992	Thai-Cambodian border	- Thai government opened the border and allowed people to enter Cambodia for gem mining. More than 80% of malaria cases detected in Thailand originated from Cambodia. - *P. falciparum* was the dominant species.	Those who acquired malaria in Cambodia brought parasites with multi-drug resistance to their home areas.
1996-1997	Sakaew Province, eastern Thailand	- Epidemic of *P. vivax* resulting from movement of Thai people into forests for woodcutting. Despite increase of *P. vivax* infections, the number of *P. falciparum* infections declined due to introduction of artesunate for *P. falciparum* treatment	Sudden change of *P. vivax* : *P. falciparum* ratio.
1998	Several provinces in southern Thailand	- Malaria infection had not occurred for more than 10 years so residual house spraying had been stopped - The economic crisis resulted in reduced manpower and operational budget for the Malaria Control programmes.	Re-emergence of malaria in these areas.

Source: Malariology, 1999

1.2.1 Malariometric parameters

The following indication are regularly measured in Thailand and their time trends from 1965 to 2000 are shown in Figure 1.1

1. Annual Parasite Incidence (API) = $\frac{\textit{microscopically confirmed cases in one year}}{\textit{Total population}} x\ 1000$

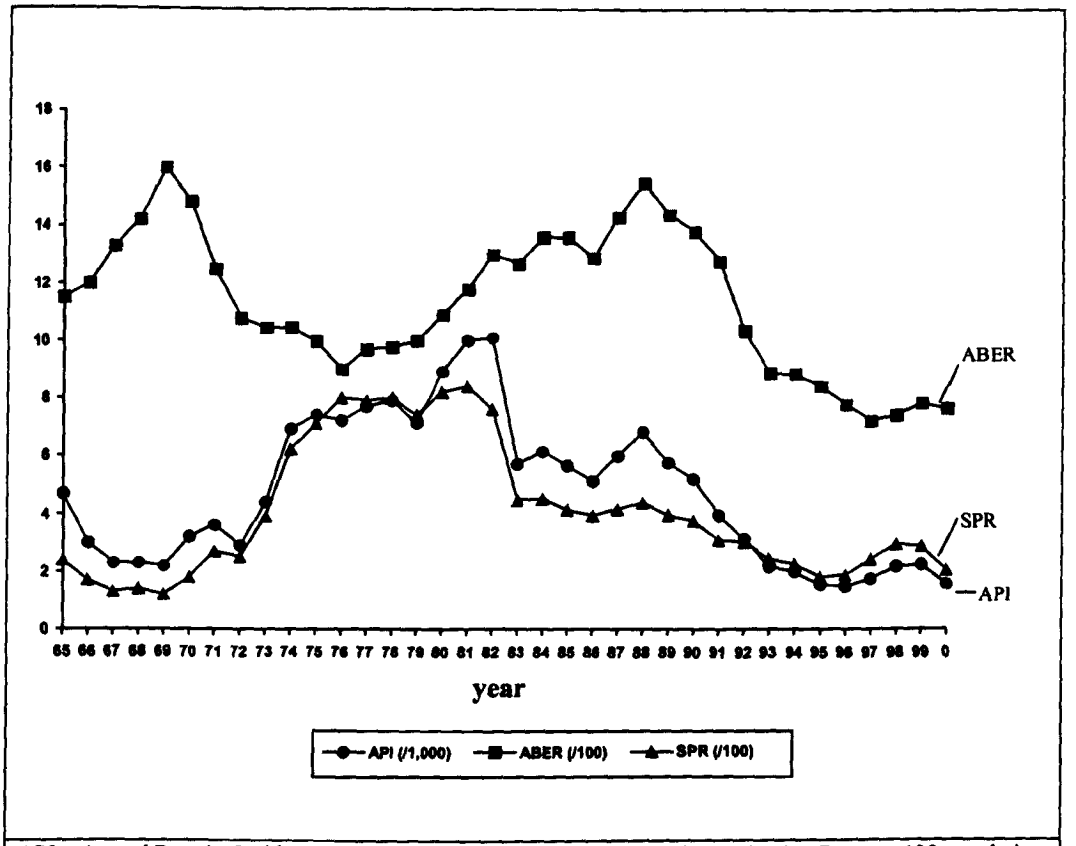
2. Annual Blood Examination Rate (ABER) = (Number of blood slides examined from all activities/total population) x 100. All of the blood slides must be taken from high risk groups amounting 3% of the population residing in malaria free areas and 10% in transmission areas.

3. Slide Positivity Rate (SPR) = $\frac{\textit{Number of positive slides}}{\textit{Total no. examined slides}} x\ 100$

1.2.2 Morbidity

The annual parasite incidence (API) from 1965-1973 was at a level of around 4.5 per 1,000 population (Figure 1.1). In 1974 the API was 6.9 per 1,000 population and then it showed an upward trend to 10.1 in 1982. One reason for this was probably emergence of resistance of *P. falciparum* to chloroquine. After that the API dramatically decreased from 10.1 in 1982 to about 5.5 in 1985. However, malaria cases slightly increased and reached a second peak in 1988 with 6.8 per 1,000 population. By the end of 1988, reported malaria cases began to decrease again, a trend that persisted through 1995. During this period malaria clinics were established throughout the country. Village malaria volunteers were recruited, resulting in early diagnosis and prompt treatment in areas of high transmission (Ketrangsee *et al*, 1989). Furthermore, quinine for radical treatment of *P. falciparum* was replaced with the new combination mefloquine/ sulfadoxine/ pyrimethamine in 1983-1984 (Malikul, 1988).

In 1997, the API was 1.8 per 1,000 persons per year, which is equivalent to approximately 99,000 malaria fever cases throughout the country, and the API then slightly increased in 1998 but slightly decreased again by the end of 1999 (Malaria Division, 1999) (Figure 1.1).

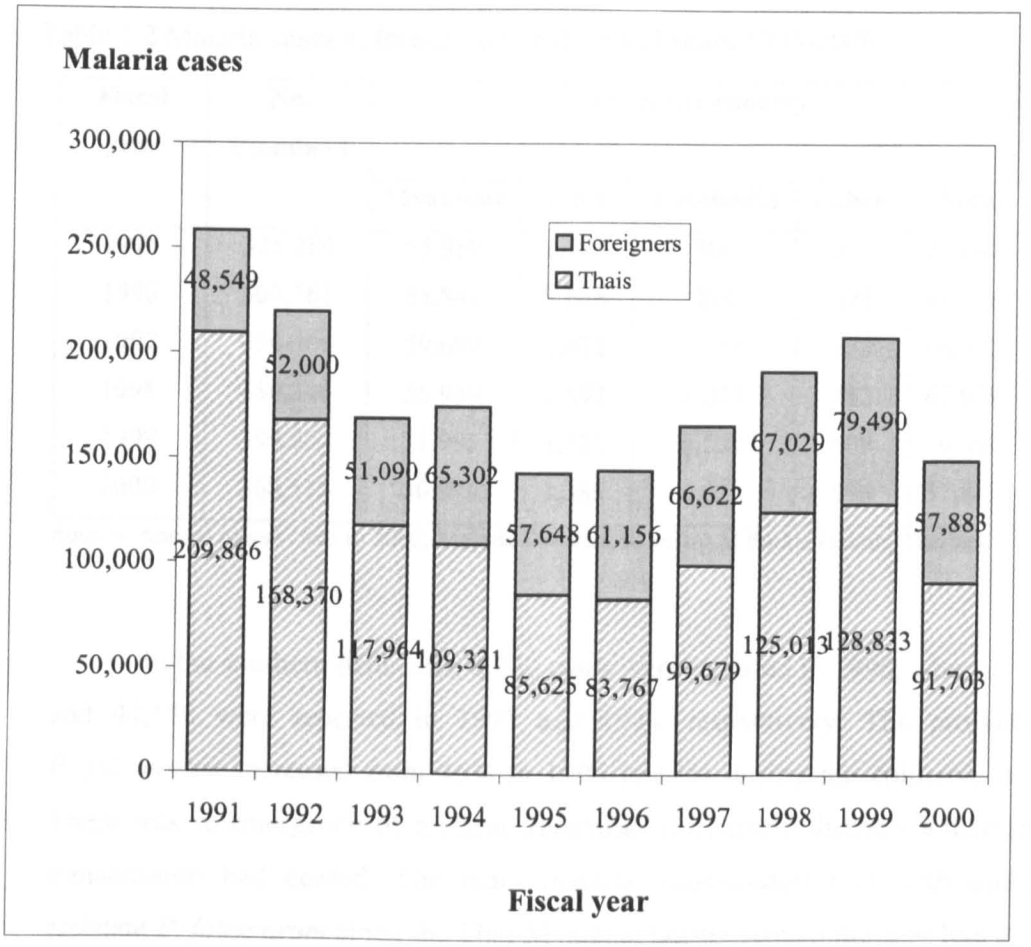


API = Annual Parasite Incidence per 1,000 population, ABER = Annual Examination Rate per 100 population
SPR = Slide Positive Rate per 100 blood smears

Source: Annual report 1994 to 2000, Malaria Division, Ministry of Public Health

Figure 1.1 Malaria cases detected by all sources in Thailand, 1965-2000

During 1997-1999 malaria epidemics were observed in various parts of the country, such as Sakaew Province along the Thai-Cambodia border which reported 613 malaria cases in 1995, 900 in 1996 and 4,800 cases in 1997. In 1998 the total reported cases was 4,200. Chanthaburi and Trat Provinces, which have long been known for multi-drug resistant malaria cases, showed a doubling of reported cases in 1998 as compared with 1996-1997. Increasing proportions of *P. vivax* were observed. In addition to Thai cases, cases among foreigners (90% of which were Burmese) have been on the increase, from 48,000 cases in 1991 to about 70,000 cases in 1999; these are mostly *P. falciparum* (more than 80%), especially cases from Cambodia dramatically increased in 1997 onwards (Malaria Division, 1997). The total number of malaria cases among Thai and foreigners for fiscal years 1991-2000 is shown in Figure 1.2 and numbers from each neighbouring countries (except Malaysia) are presented in Table 1.2.



Source: Annual report 2000, Malaria Division, Ministry of Public Health, Thailand

Figure 1.2 Malaria cases among Thai and foreign nationals for the whole country in fiscal years 1991-2000

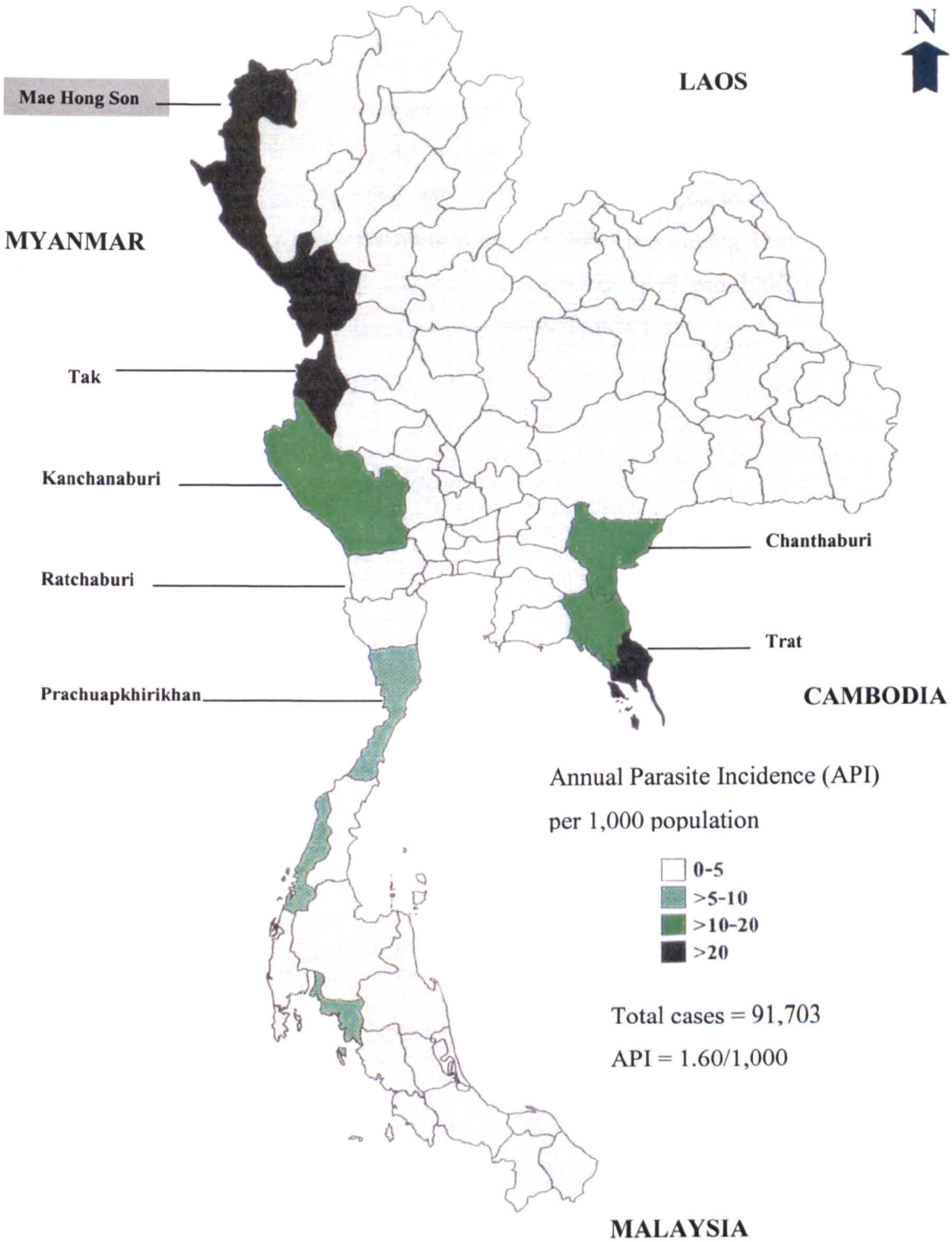
Table 1.2 Malaria cases in foreign nationals, fiscal years 1995-2000.

Fiscal year	No. examined	Cases per country					SPR (%)
		Myanmar	Laos	Cambodia	Others	Total	
1995	328,210	55,989	1,141	306	212	57,648	17.56
1996	307,761	58,841	1,648	294	373	61,156	19.87
1997	450,406	59,699	2,472	3,718	733	66,622	14.79
1998	450,396	56,939	1,592	8,015	483	67,029	14.88
1999	399,867	71,995	1,321	5,532	609	79,490	19.88
2000	368,513	50,976	1,385	4,926	596	57,883	15.71

Source: Annual report 1995 to 2000, Malaria Division, Ministry of Public Health, Thailand

In the southern peninsula, 8,730 cases were reported in 1996, whereas 13,620 and 47,150 were reported in 1997 and 1998, respectively. The proportion of *P. falciparum* increased from 45% in 1996 to 56% during the epidemic in 1998. There was re-emergence of malaria transmission in many districts where malaria transmission had ceased. The main malaria transmission foci with multi-drug resistant *P. falciparum* along the Thai-Myanmar border remain more or less stable. It is anticipated that malaria epidemics will continue and the Control Programme may take a few years to overcome the problems of the type which have been outlined and bring down the national malaria incidence to an acceptable level.

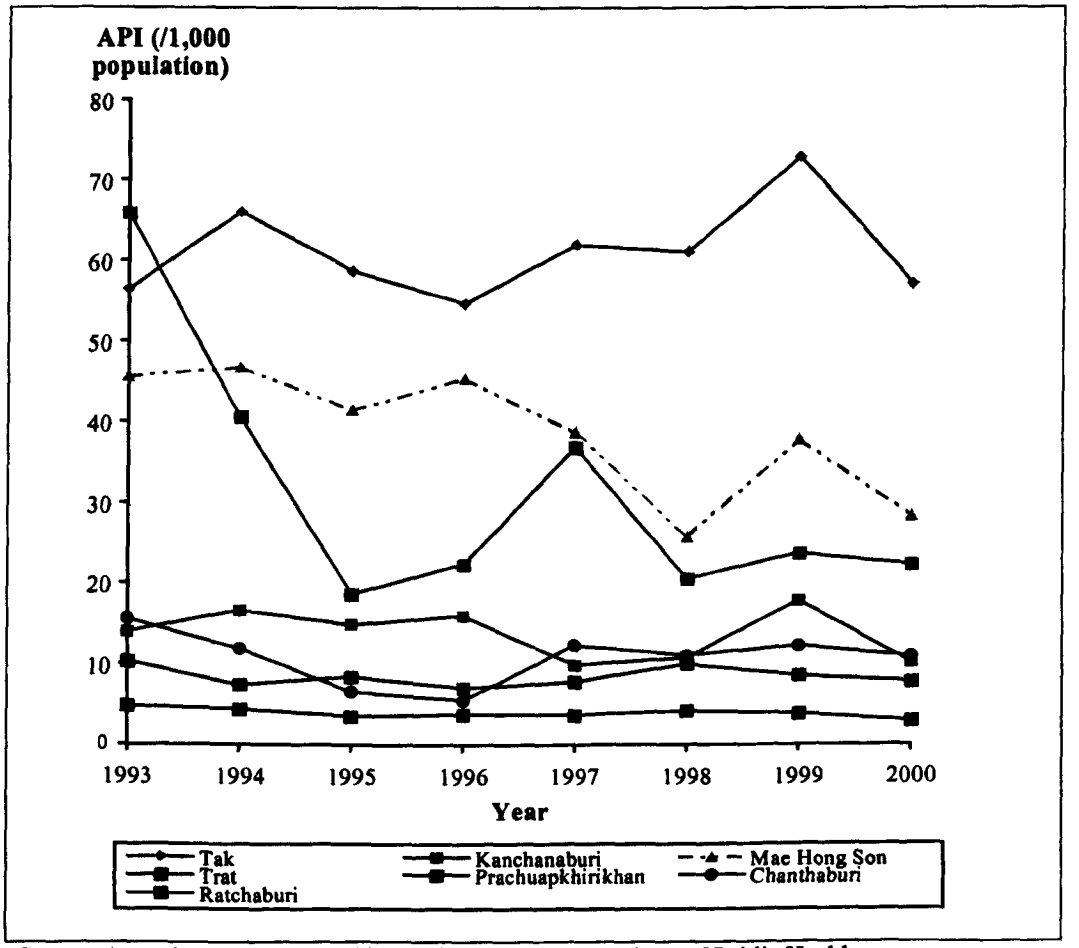
In the highly endemic areas that border with Myanmar in the Northwest and Cambodia in the east, provincial APIs range from about 30 to nearly 50 per 1,000 persons per year (Malaria Division, 1997). Figure 1.3 shows the API by province in 2000.



Source: Malaria Division, Department of Communicable Diseases, Ministry of Public Health

Figure 1.3 Distribution of Annual Parasite Incidence by Province, 2000

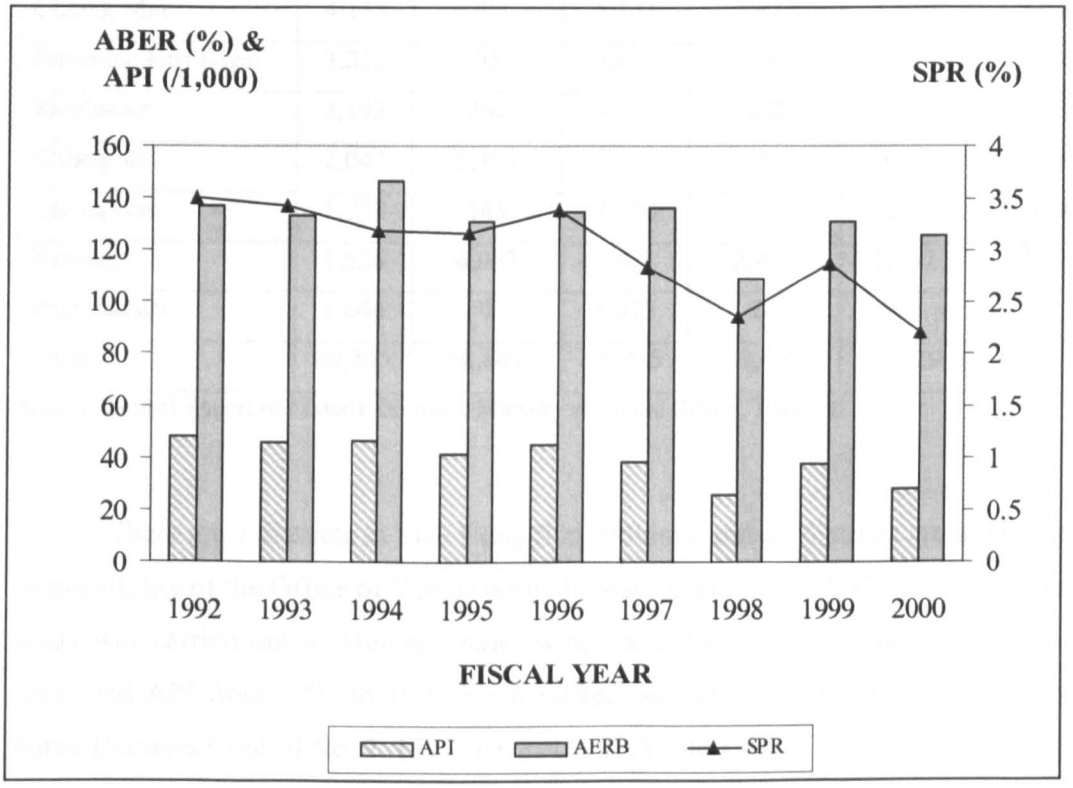
The present project was conducted in Mae Hong Son Province, situated in the Northwest near the Myanmar border. Since 1994 this province has had the second highest ranking for Annual Parasite Incidence (API) among the seven border provinces of Thailand. The API of this province ranged from 38 to 47 per 1,000 persons per year, corresponding to about 8,000 cases among Thai nationals in most years since 1993, but the API was under 30 in 1998 and 2000, corresponding to about 5,000 cases (Malaria Division, 1997) (Figure 1.4).



Source: Annual report 1994 to 1999, Malaria Division, Ministry of Public Health

Figure 1.4 Malaria incidence in seven border provinces in Thailand from 1993-2000. Their location is shown in Figure 1.3.

The Annual Blood Examination Rate (ABER) of Mae Hong Son province was 136 to 146 percent from 1992 to 1994 and slightly decreased to 125 in 2000. The Slide Positivity Rate (SPR) has been about 3% (Figure 1.5) (Malaria Division, 1994; 1995; 1996; 1997; 1998; 1999; 2000). From 1996 to 1998, this province has had the third highest numbers of malaria cases among the 10 provinces along the Thai-Myanmar border (Table 1.3).



Source: Annual Report of Malaria Division (1995-2000), Ministry of Public Health, Thailand

Figure 1.5 API (Annual Parasite Incidence per 1,000 population), ABER (Annual Blood Examination Rate per 100 population) and SPR (Slide Positive Rate per 100 blood smears) in Mae Hong Son Province, fiscal years 1992-2000

Table 1.3 Malaria cases among Thai and foreign nationals in 10 provinces along the Thai-Myanmar border (1996-1998)

Year	1996		1997		1998	
Border area	Thais	Foreign nationals	Thais	Foreign nationals	Thais	Foreign nationals
Tak	22,432	45,336	25,751	43,014	24,925	38,740
Kanchanaburi	11,607	3,524	7,249	3,981	7,996	5,037
Mae Hong Son	**8,879**	**2,134**	**7,639**	**3,901**	**5,186**	**2,801**
Chiang Mai	4,118	701	5,141	1,872	3,682	2,010
Prachuap Kiri Khan	3,235	95	3,651	350	4,570	834
Ratchaburi	3,143	462	3,044	1,527	3,623	2,360
Chiangrai	2,042	2,393	791	2,343	901	1,997
Chumporn	1,737	143	2,120	232	2,528	164
Ranong	1,526	4,053	1,678	2,455	2,467	2,996
Phetchaburi	1,646	0	1,375	24	951	0
Total	60,365	58,841	58,435	59,699	56,829	56,939

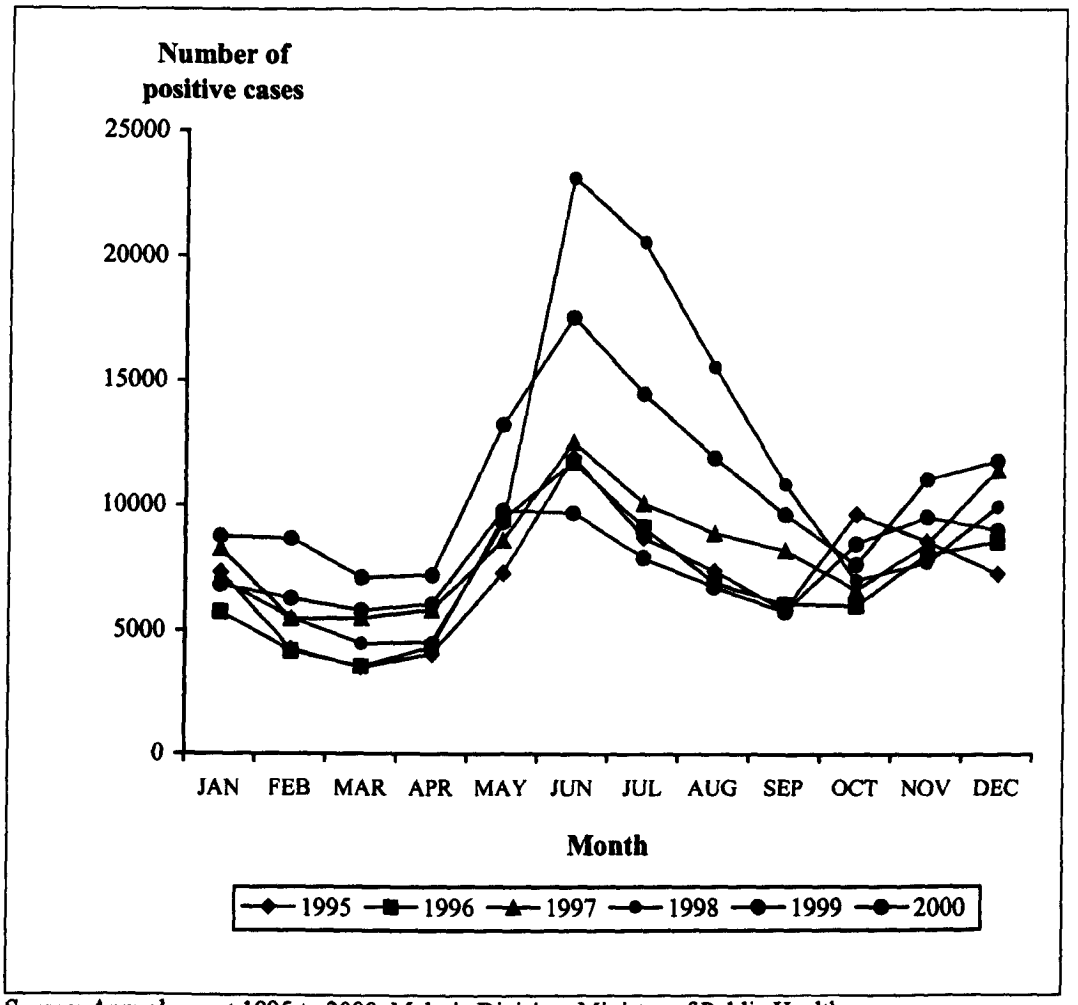
Source: Annual Report of Malaria Division, Ministry of Public Health, Thailand

There are 7 districts in Mae Hong Son Province and all districts are under the responsibility of the Office of Vector-borne Disease Control No. 2, Chiang Mai. This study was carried out in Muang district which had the highest number of positive cases and API from 1997 to 1999 but it ranked second in 2000 (Office of Vector-borne Diseases Control No. 2, 1997; 1998; 1999) (Table 1.4).

Table 1.4 Malaria incidence in 10 problem districts under the responsibility of the Office of Vector-borne Diseases Control No. 2, Chiang Mai, 1997-2000.

Problem districts	1997		1998		1999		2000	
	Cases	API /1000	Cases	API /1000	Cases	API /1000	Cases	API /1000
Muang Mae Hong Son	3,444	88.2	1,573	62.6	2,100	57.5	1,494	30.9
Mae Sariang	1,376	29.5	1,442	45.6	1,630	57.8	1,391	38.5
Sobmoey	971	36.8	795	28.3	1,558	32.8	1,011	38.5
Chiang Down	945	12.8	787	11.7	1,467	19.4	760	9.8
Mai Ai	686	10.2	632	8.4	965	36.0	755	16.7
Om Koi	546	12.6	546	12.3	689	15.3	669	23.0
Pai	506	20.7	499	16.2	563	9.7	518	26.8
Muang Chiang Mai	494	2.0	494	1.9	592	8.4	313	5.0
Khumyaum	496	25.8	398	21.6	525	27.6	274	20.8
Phangmapha	468	37.5	293	23.0	468	7.5	266	3.9

In Thailand malaria occurs throughout the year with two seasonal peaks, one at the beginning of the rainy season (May-July) and the other during the winter (November-January) (Figure 1.6). Malaria transmission in northern Thailand follows the same pattern.

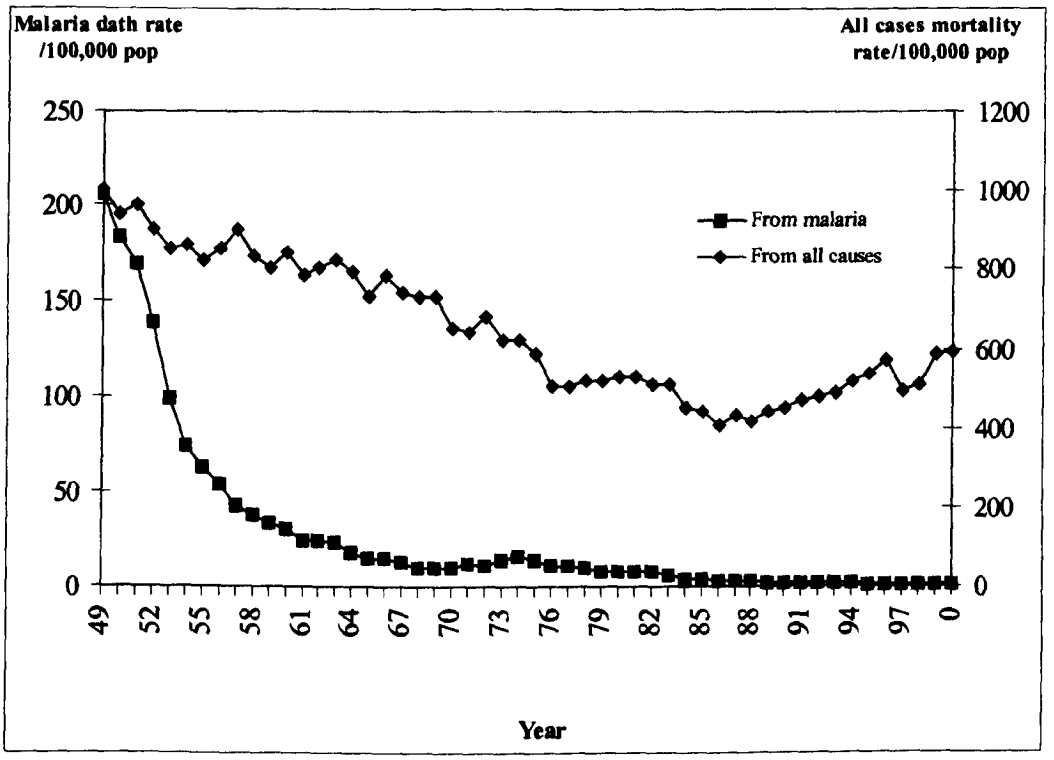


Source: Annual report 1995 to 2000, Malaria Division, Ministry of Public Health

Figure 1.6 Monthly malaria cases in Thailand, fiscal years 1995-2000

1.2.3 Mortality

Malaria was formerly a leading cause of death in Thailand, with a mprtality rate as high as 351 per 100,000 population in 1947 (Malikul, 1988). Over the last three decades, the malaria death rate has dropped from 15.2 in 1965 to 10.1 in 1970 and it has continued to decrease to a rate of 1.0 death per 100,000 population in 2000 (Ministry of Public Health, 1966; Pinichpongse, 1986; Malaria Division, 2000) (Figure 1.7).



Source: Annual report 1994 to 2000, Malaria Division, Ministry of Public Health

Figure 1.7 Malaria Mortality Rate in Thailand 1965-2000

1.2.4 Malaria control programme in Thailand

The Anti-Malaria Programme has been operating for over 50 years. This programme is the responsibility of the Malaria Division, Ministry of Public Health. The control strategy consisted of residual insecticide house spraying with DDT (Dichloro-diphenyl-trichloroethane) and chemotherapy. DDT was first used in Chiang Mai Province, Northern Thailand in about 1950, beginning as a pilot project for mosquito control supported by WHO/UNICEF (Hongvivatana *et al.*, 1982). Based on the highly successful field trials, DDT became the insecticide of choice for mosquito vector control in the country. In 1965, malaria eradication was adopted as the target within the framework of the National Malaria Programme. In 1979, a revised Anti-Malaria Programme was developed to provide a comprehensive control programme to all at-risk populations in Thailand, and DDT was again specified as the primary insecticide for indoor-residual spraying (IRS).

Currently, under the 8th National Plan (1997-2001), the aim has been to bring down the national malaria incidence rate to 1, or less, per 1,000 population and the malaria mortality rate to 0.4, or less, per 100,000 population by 2001. Furthermore, the incidence rate in the 30 border provinces should have been reduced to 3, or less, per 1,000 population by 2001 (Malaria Division, 1997).

The control operations are broadly divided into two categories (areas with continuing malaria control and areas where malaria has been eradicated). Regarding the control areas, the aim is for long-term malaria control in the forested and hilly areas of the country and prevention of the re-establishment of malaria transmission in the lowlands. The malaria eradicated areas include the major part of the country and they are no longer covered by routine vector control activities using DDT (Malikul, 1988). These areas may be more precisely defined as follows (Chareonviriyaphap *et al.*, 2000) :-

1. Control areas: all areas receiving or needing active control and surveillance activities. These areas principally consist of hilly, forested areas, rubber

plantations, mountainous areas, most border areas and other endemic areas in the country.

1.1. Transmission areas:

1.1.1 Perennial transmission areas (designated as A1): where transmission occurs year-round or at least for 6 months of the year. Approximately 1.5 million people live within these areas.

1.1.2 Periodic transmission areas (A2): where transmission is reported for five months or fewer per year. Approximately 2.3 million people live within these areas.

1.2 Non- transmission areas

1.2.1 High risk areas (B1): transmission has not been reported within the last three consecutive years but primary and secondary vectors are found. Consequently, these areas are potentially suitable for malaria transmission (high and moderate receptivity). Approximately 11.4 million people live within these areas.

1.2.2 Low risk areas (B2): transmission has not been reported within the last three consecutive years and neither primary nor secondary vectors are found. Suspected vectors, however, may be found. Approximately 25.7 million people occupy these areas.

2. Pre-integration areas: transmission is no longer occurring. These areas have been categorized as low risk for at least 3 years and local health services, such as hospitals and health centres, are able to perform case detection, treatment and case investigation. Approximately 4.4 million people reside in these areas.

3. Integration areas: areas that have been in the pre-integration category for at least 3 years and the Provincial Health Offices are capable of managing all activities concerning malaria. These areas contain a population of 10.8 million.

In the forested hills and mountains and border areas malaria is still the most important public health problem and control of transmission has faced many difficulties, for example lack of acceptance by the local people of DDT house spraying and failure to protect mobile populations (Ketrangsee *et al.*, 1991). In these areas health education to reduce risky behaviour is unsuccessful. Moreover, epidemics and morbidity occur in highly receptive areas due to migration of non-immune workers to development projects, and influx of tourists.

The work described in this thesis took place in an area of northern Thailand which falls into the A1 category defined above. Controlling malaria in this area is difficult, with many obstacles such as many favourable breeding places for the mosquito vectors, the movement of hill tribes for agricultural and forest related activities, movement across the border, and difficulties of transportation. Singhanetra-Renard (1986) carried out a study in this area and she found that malaria transmission is associated with illegal activities that expose villagers to malaria vectors in the forest, or which bring large number of infected workers from Myanmar into the country.

1.2.4.1 The acceptance of the policy of using house spraying and use of impregnated bednets

In order to achieve the objectives of the 8th National Plan, the routine activities of the malaria control programme are supposed to include spraying of houses every 6 months, and the annual permethrin treatment of bednets. To be successful, all programmes require a high degree of public participation. However, the rate of acceptance by the people of residual spraying with 75% WDP (Water Dispersible Powder) of DDT is quite low (Prasittisuk, 1985). Because of its odour and staining, and because some villagers think that DDT can cause death of their animals, spraying has increasingly been refused. In addition some hill tribes believe that it disturbs their evil spirits.

In the late 1970s entomologists started using synthetic pyrethroids, with high insecticidal activity and low mammalian toxicity. A study in 6 villages in Muang District, Mae Hong Son Province showed that 95% of those sampled accepted deltamethrin spraying, and 84% thought that it was better than DDT and were willing to accept the next deltamethrin spraying (Prajakwong, 1997). Because of changing human response to spraying and the possible adverse long-term impact of DDT on the environment, the use of this insecticide in vector control is being slowly replaced with various synthetic pyrethroids, such as deltamethrin, permethrin and lambda-cyhalothrin. For the reasons mentioned above, DDT purchase was stopped in 1995. However, the remaining stock of DDT is still being used in malaria problem areas of Thailand and it still serves as the primary chemical for mosquito vector control. Several studies indicate that chemical insecticides still have an effect on malaria vectors in Thailand (Ismail *et al.*, 1975; 1978; Prasittisuk, 1985; Chareonviriyahpap *et al.*, 1999).

The long-term intensive use of insecticide has led to the development of insecticide resistance in many countries. Unfortunately, reliable information on vector resistance patterns in Thailand is limited. Bang (1985) reviewed the insecticide resistance among known malaria vectors in Thailand. He showed that resistance to DDT and other chemicals is minimal. A study of encapsulated fenitrothion used against *Anopheles. minimus* in Prae Province, Thailand, suggested that residual spraying at the beginning of the dry season was effective for at least 4 months after the spray round and the study also suggested that this species was sensitive to fenitrothion (Suwonkerd, 1997).

One approach which has received much attention all over the world during the past ten years is the use of pyrethroid-impregnated bednets (World Health Organisation, 1989). This approach is important in reducing malaria morbidity and mortality, especially in Africa (Lengeler *et al.*, 1996). These nets can reduce man-vector contact by acting both as a physical barrier by protecting the sleeper from mosquito bites and as a chemical barrier by repelling mosquitoes from the sleeper and also killing mosquitoes that contact the nets and hence reducing the vectorial

capacity of the mosquito population (Snow *et al.*, 1987). A large number of trials with insecticide-treated bednets has been carried out in various parts of the world (Rozendaal and Curtis, 1989; Lengeler, 1998). In Vietnam, the most effective vector control measure is the use of insecticide-treated bednets (ITN). People have accepted the method and socialised application has quickly become widespread, with 11 million people having their nets treated annually (Tran Duc Hinh, 2001). Field trials have evaluated the impact of community-wide use of pyrethroid-treated bednets on anopheline vectors and malaria transmission (Curtis, 1996). The use of impregnated bednets can reduce child mortality; a study in Kenya showed a significant reduction in severe life-threatening malaria attacks among children from the treated net villages (Nevill *et al.*, 1996). Several studies from Tanzania showed that after introduction of treated bednets there were reduced man-biting, parity and sporozoite inoculation rates as compared with rates estimated from people without ntes and this represents the "mass effect" of wide spread use of insecticidal nets (Magesa *et al.*, 1991; Msuya and Curtis, 1991; Njunwa *et al.*, 1991; Curtis *et al.*, 1992). A study on the effect of community-wide use of bednets treated with lambdacyhalothrin in Sierra Leone showed that the parous rates were significantly reduced in all intervention villages, but the sporozoite rate fell in only some of the villages with treated nets (Magbity *et al.*, 1997). These results were different from studies from The Gambia (Lindsay *et al.*, 1989; Quiñones, *et al.*, 1998), Kenya (Mbogo *et al.*, 1996) and Thailand (Aramrattana 1993; Somboon *et al.*, 1995), where treated bednets did not seem to reduce the mosquito survival, outdoor biting rate, sporozoite rate or human blood index. The inconsistency of results in different areas indicates that the effect of bednets on different populations of *Anopheles* depends on local circumstances.

The study of Sri-aroon *et al.* (1998) in the Western part of Thailand showed that 76.4% of mothers or guardians used impregnated bednets regularly to protect their children, whereas 23.6% used them infrequently and a few never used them. Moreover, they showed that the use of impregnated bednets was significantly related to factors such as knowledge of malaria prevention, perception of benefits of their use, and the receipt of information about impregnated bednets from malaria workers.

Luxemburger *et al.* (1994) showed that children in refugee camps on the Thai-Myanmar border who slept under treated bednets experienced fewer *falciparum* malaria infections and clinical attacks than those who slept under untreated nets. Although impregnated bednets have a high potential efficacy against malaria, there are some factors that may prevent the realisation of their potential. In six villages in northern Thailand it was found that, although about 82 % of households in the villages owned mosquito nets, only 70% of villagers working outside the villages carried them to the forest when there was a low density of mosquitoes. Some people did not consider it worthwhile to sleep under a net (Chitprarop, 1986). Another problem with bednet use is that the number of mosquito nets in a house is often not enough for the number of persons in the family. Butraporn *et al.* (1995) has reported that the average number of mosquito nets per family was 2.1, and an average family size was 4.8 persons. So approximately 2-3 persons shared a net which would mean that at least one person would be likely to sleep uncovered by a net and hence be accessible to mosquito bites. Only 34% brought their net with them when they travelled for an overnight stay outside their home village.

1.2.4.2 Population movement

In Thailand, population mobility in the forested foothill areas contributes to active malaria transmission, and conventional methods of control are often found to be impractical (Sommani *et al.*, 1983; Butraporn *et al.*, 1986; Singhanetra-Renard, 1986; 1993). Occupational and labour movement for gem mining, logging etc. along the Cambodian and Myanmar borders is an important factor in current malaria problems in Thailand. It is considered to have contributed to significant increases of *P. falciparum* morbidity and the high levels of resistance to many anti-malarial drugs (Radford *et al.*, 1976; Gogoi *et al.*, 1996). The problem of population movements in forest areas is connected with the fact that the most efficient South East Asian vectors, *An. dirus* and *An. minimus*, are forest species, unlike for example *An. gambiae* in Africa which is well adapted to life in villages. Aramrattana (1993) described the changing patterns of malaria epidemiology of people who lived in villages in the Northwest of Thailand according to geographical area, age, sex, and

occupation, emphasising the importance of movement of people in and out of villages. He showed that malaria incidence was directly related to the number and duration of journeys. The risk of catching malaria during a journey was at least 7.8 times higher than in the static population. The association of *falciparum* malaria with migration contributes to a peak of incidence in young adults who are more mobile as compared to the rest of the population (Aramrattana, 1993; Luxemburger *et al.*, 1996). Moreover, this age group appeared to be less compliant with any protective measures against malaria during the time they are based in the forested areas (Butraporn *et al.*, 1995).

1.2.4.3 Surveillance system

The Roll Back Malaria programme of WHO emphasises provision of early diagnosis and prompt treatment, selective and sustainable preventive measures (including vector control), and early detection of epidemics. For these to be effective surveillance is essential (World Health Organisation, 1997). The primary purpose of surveillance is to guide malaria control activities by producing a numerical picture of trends in malaria incidence and mortality in the various units that diagnose and treat malaria.

From the 1970s until recently, the lag between the day of blood sampling and day of radical treatment was considered a major problem since presumptive treatment with chloroquine ceased to be useful in the 1970s. The Thai control programme solved this problem by establishing a network of malaria clinics. This was a major investment in terms of expensive equipment (microscopes) and the training of hundreds of house visitors to become microscopists. It took a number of years before the malaria clinic system could be expanded to remote and highly malarious areas throughout the country. However, the number of malaria clinics has increased from 174 in 1979 to 526 in 2000. There were about 38,700 village malaria volunteers in 1989 and then the number dramatically decreased to 15,810 in 2000 (Malaria Division, 1989; Ketrangsee *et al.*, 1991; Malaria Division, 2000). There are two types of volunteer working with the malaria programme, village health

volunteers (VHV) serving under the Office of the Permanent Secretary of state of the Ministry of Public Health and village malaria volunteers (VMV) serving under the malaria Division. Some volunteers serve as both a health and malaria volunteer. The numbers mentioned above refer to VMV. The reason for decreasing numbers of VMV is the switching of VMV to be VHV especially in villages where the malaria prevalence is that under the control of malaria staff. Another reason is there was no replacement of VMV when they resigned or died.

At the central level in Thailand malaria cases are recorded by the Malaria Division, Department of Communicable Disease Control, Ministry of Public Health, through a mechanism of active case detection (ACD) and passive case detection (PCD). In the past there was house to house visiting but now that has been withdrawn and replaced by "Special Case Detection" (SCD) which is active case finding carried out during the peak transmission season or epidemics to supplement data from villages that are inaccessible to PCD. The operation of mobile malaria clinics is another activity similar to SCD but with facilities for microscopy. The malaria surveillance system in Thailand is summarised in Figure 1.8.

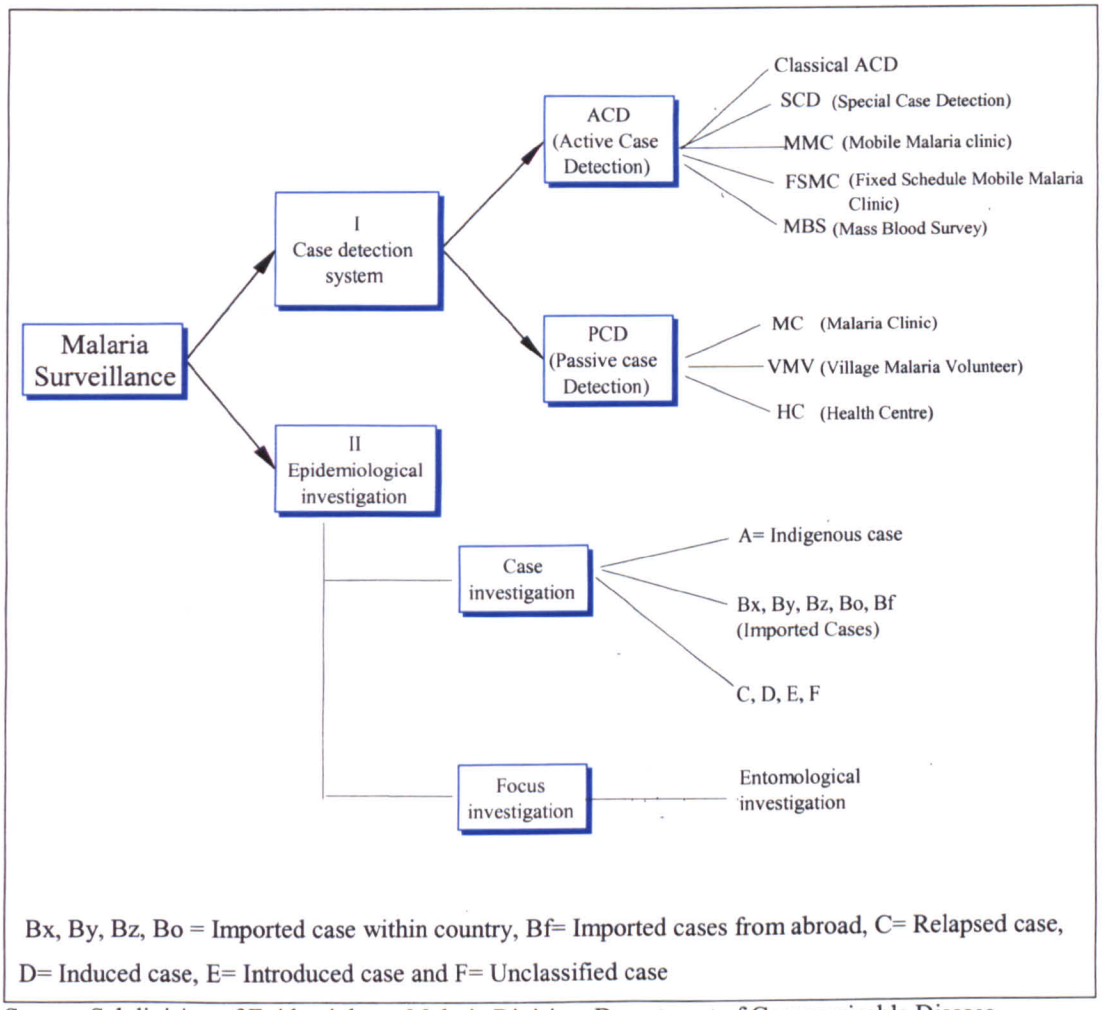
At the country level the programme comprises five regions called Offices of Vector-borne Disease Control (VBDO), formerly the Malaria Centres. There are 39 Vector-borne Disease Control Centres (VBDC) at the provincial level and 302 Vector-borne Disease Control Units (VBDU) and 536 Malaria Clinics at district levels (Figure 1.9). In rural settings a VMV is expected to visit each house at fortnightly intervals, find cases of fever since the time he last visited, and collect blood smears from suspected cases. He then gives presumptive treatment and makes appropriate entries in his register. Blood smears collected during the domicilliary visits are delivered to a laboratory technician at the health centre or given to a malaria worker from the VBDU. A laboratory technician examines blood smears and then the malaria worker will go back to the village for case investigation and to give anti-malarial drugs to those people who are slide positive, or leave the drug with the VMV if the patients are absent. In urban areas there is no active case detection except under special circumstances, but there are VBDU attached to the hospitals in

certain areas. Data from PCD reflects those patients who go to hospitals, rural health centres, or VBDU for blood examination. Those found positive for malaria are given anti-malarial drugs. In both PCD and ACD, most of the malaria films are taken only from subjects with fever or who have had a history of fever (or subjects with a history of movement to malarious areas in Thailand), hence only the symptomatic population is sampled (Pull, 1972; Fox *et al.*, 1987). PCD can be very effective if there is a good coverage. Mills (1987) has argued that ACD is not cost-effective because blood smears are only taken from fever cases so it will not detect asymptomatic malaria. To detect asymptomatic malaria mass blood surveys (MBS) are required. The research described in this thesis has bearing on the question of whether PCD or ACD would detect and lead to the treatment of the reservoir of *Plasmodium*, which infects the vectors.

There are some biases and errors in the routine data collection which tend to underestimate parameters such as API. Epidemiological information is supposed to be obtained by interviewing cases. However, sometimes the case cannot be found, and malaria personnel have to interview neighbours who may give inaccurate information. Moreover, API may be underestimated if the collections of blood slides do not cover every village, especially the villages which cannot be accessed during the rainy season. It is generally considered that these sources of error remain reasonably constant from year to year so that changes in incidence between years are correctly indicated by the available data. Likewise, the ABER comprises data from each one of the surveillance activities, but again, it is assumed in this thesis that should biases exist, they would be approximately constant with time.

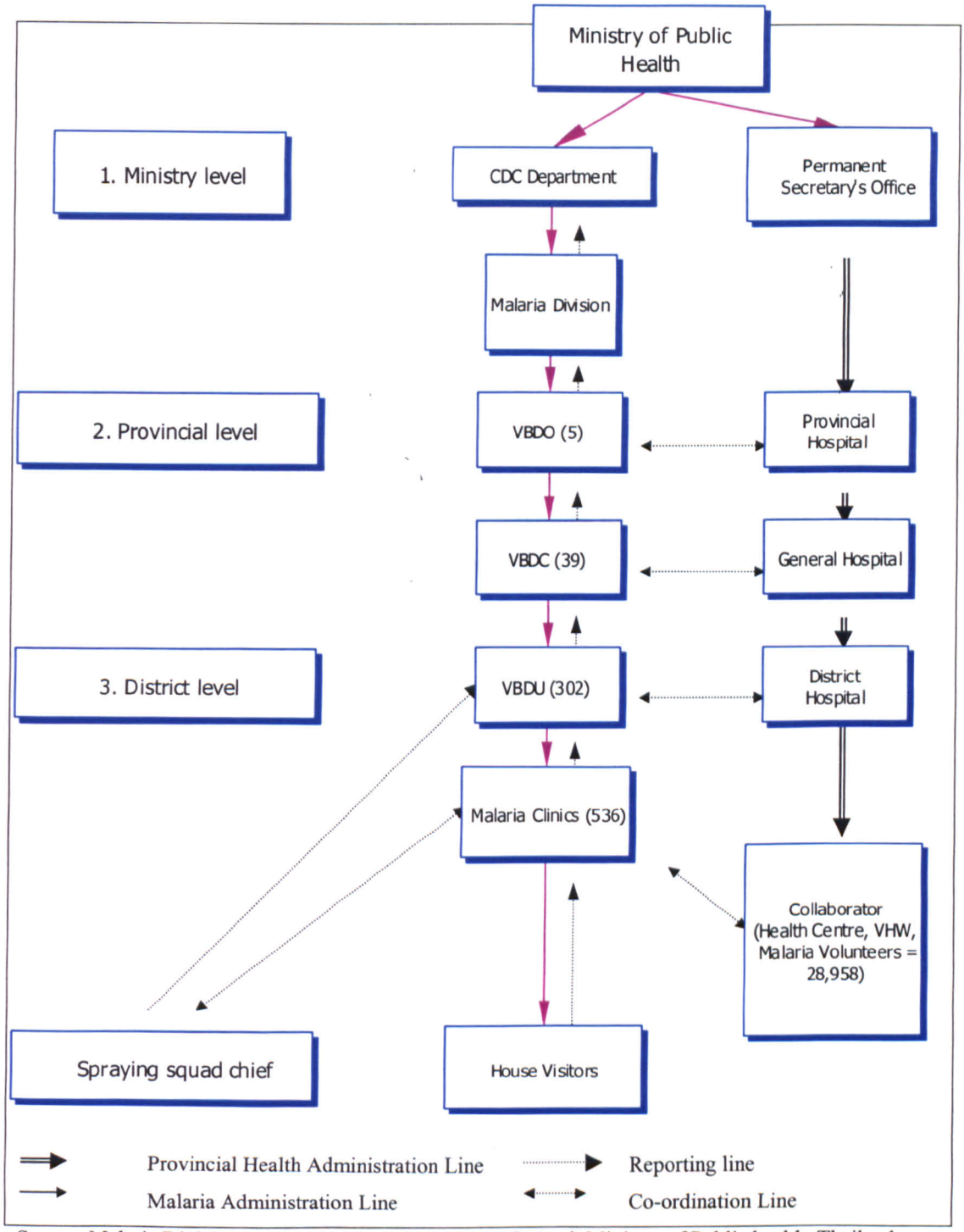
Of the staff who were involved in this project, four were from VBDU No. 8 and one driver was from VBDC No.21, Mae Hong Son Province. Normally, blood collection is carried out twice a month by the staff from VBDU No. 8, especially in the villages where the malaria prevalence is high. Thus, before starting the project, we had arranged with the head of this office to set up a schedule related to their routine work. Then the head of the unit made available two blood collectors, two microscopists and one driver for each survey. In this project, we asked the malaria

health volunteers to announce the date and time of blood collection via a loud speaker tower in each village. After examination the people who were slide positive were called back for treatment and if they were age>15 years then they were asked for mosquito feeding.



Source: Subdivision of Epidemiology, Malaria Division, Department of Communicable Disease Control, Ministry of Public Health, Thailand

Figure 1.8 Malaria Surveillance System in Thailand



Source: Malaria Division, Dept Communicable Disease Control, Ministry of Public health, Thailand

Figure 1.9 Organisation and reporting chart of Malaria Surveillance System in Thailand

1.3 The Parasites

Malaria is transmitted by female *Anopheles* mosquitoes. The mosquito becomes infected with malaria parasites when it takes blood to nourish its eggs from a person whose blood contains the sexual forms of the parasite (gametocytes). Female macrogametocytes and male microgametocytes ingested in this way become mature gametes in the midgut of the mosquito. The male gametocyte produces 8 flagellated gametes in a sudden, violent exflagellation which takes only a few minutes at the mosquito gut. Fertilization of a female gamete (macrogamete) by a male gamete (microgamete) results in a motile zygote (ookinete) which migrates through the gut wall and develops into an oocyst. Asexual division inside the oocyst yields as many as 10,000 elongated, spindle-shaped sporozoites which are released when the oocyst eventually ruptures. The sporozoites migrate via the body cavity to accumulate in the salivary glands of the mosquito. When the infected female *Anopheles* takes subsequent blood-meals, the sporozoites are inoculated into the bloodstream of the human host because the mosquito pumps in saliva, containing anti-coagulants while it is sucking up blood. The sporozoites are carried in the blood to the liver, where they invaded the parenchymal cells and develop into exo-erythrocytic schizonts. The life cycle of *Plasmodium* is shown in Figure 1.10.

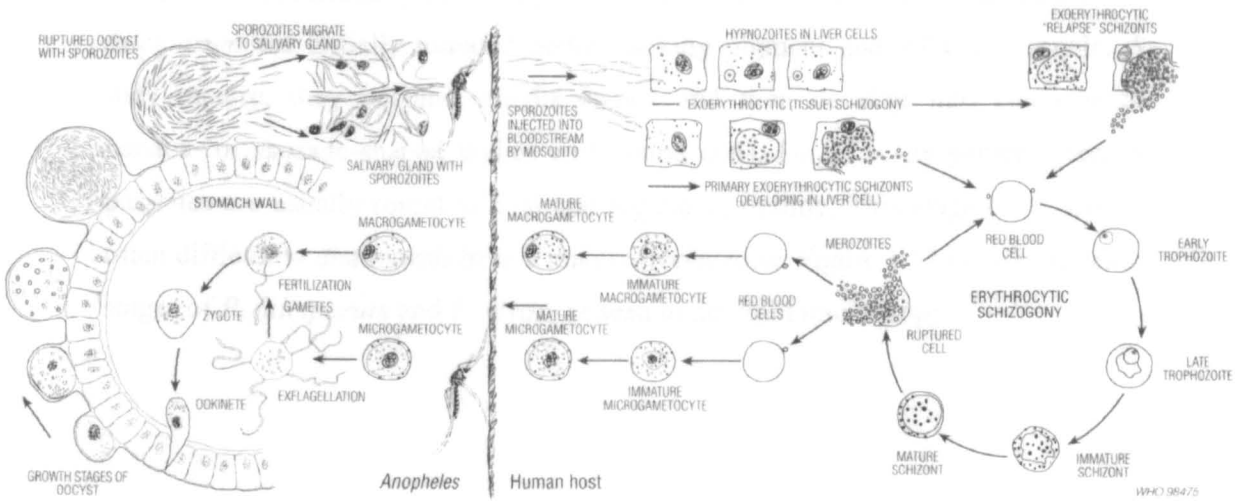


Figure 1.10 The life cycle of *Plasmodium* (from Gilles and Warrell, 1999)

Once a patient is infected with *Plasmodium*, it takes 7-27 (average 12) days for *P. falciparum* and 13-17 days for *P. vivax* for the first clinical signs to appear (pre-patent period). If the patient lacks immunity, infection may quickly develop into an acute form. In blood films the microscopist usually sees only young trophozoites (ring form) (Figure 1.11). The young ring forms of *P. falciparum* are very small, measuring about one-sixth of the diameter of a red blood cell. There are two chromatin granules in many of the ring forms. There are frequently several ring forms to be seen in a single host cell. The ring form and older trophozoites usually disappear from the peripheral circulation after 24 hours and they are found in the capillaries of the internal organs such as the brain, heart, spleen etc. In *P. vivax*, trophozoites are very large - about one-third the diameter of the red blood cell. They

contain of prominent red chromatin dots (*Schüffner's* dots) and a fine circle of blue cytoplasm. Occasionally there may be two chromatin dots. The sexual stages of *P. falciparum* are initially rounded bodies lacking pigment and with no vacuole. As they mature, they become spindle-shaped and then develop into characteristic banana- or sausage-shaped bodies with round ends. For *P. vivax* gametocytes, the parasites are usually round to oval and regular in outline. This stage of *P. vivax* is often difficult to distinguish from a mature trophozoite. Figure 1.12 shows the sexual stages of *P. falciparum* and *P. vivax*, as seen under light microscopy.

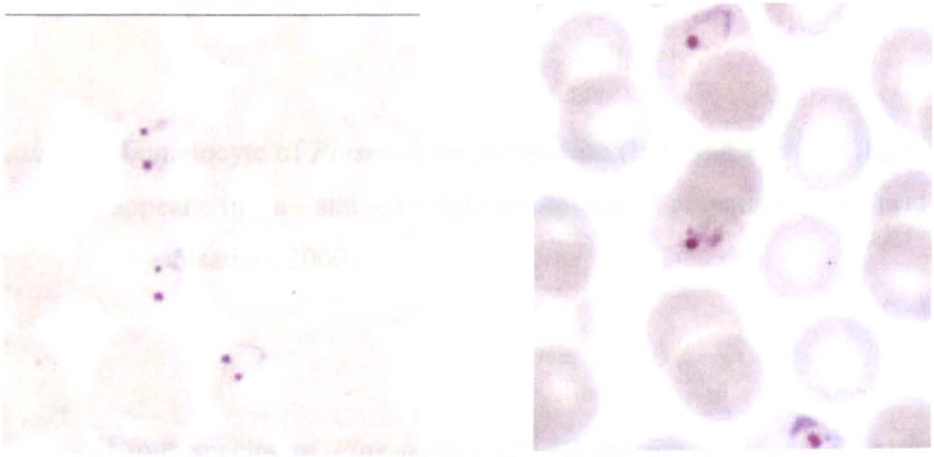


Figure 1.11 Erythrocytic trophozoites, as seen using light microscopy *P. falciparum* (left) and *P. vivax* (right) (World Health Organisation, 2000)

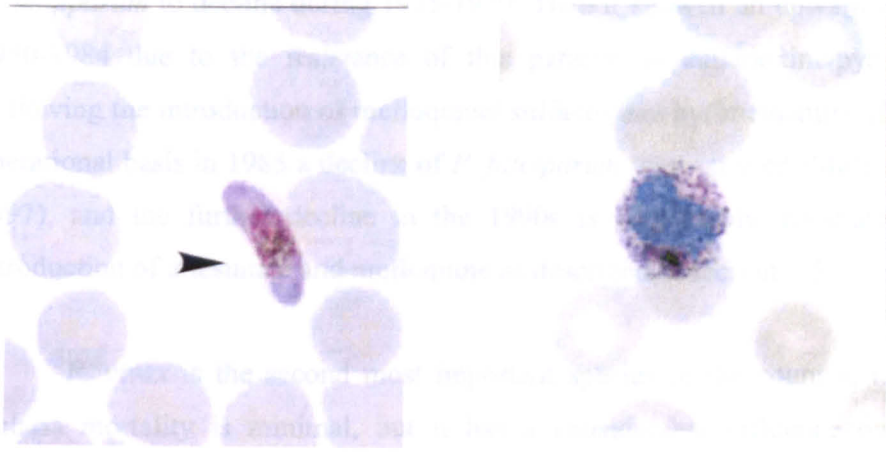


Figure 1.12 Gametocyte of *Plasmodium falciparum* (left) and *P. vivax* (right), as they appear in a stained light-microscope preparation (World Health Organisation, 2000).

All four species of *Plasmodium* (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*) which cause human malaria, have been reported in Thailand (Snounou *et al.*, 1993c; Zhou *et al.*, 1998). The distribution and importance of each, however, varies greatly in relation to the overall epidemiology of malaria. *P. falciparum* and *P. vivax* are the most important, *P. falciparum* is widely distributed and responsible for most of the epidemics and deaths from malaria in the country. The proportion of *P. falciparum* and *P. vivax* over the period 1965-2000 is showed in Figure 1.13.

The proportion of *P. falciparum* has always been higher than the other species, and this was especially true during the period 1967-1974 (70%-80%) due to the emergence of chloroquine resistant *falciparum* malaria and the lack of an adequate substitute during that time (Harinasuta *et al.*, 1962; Harinasuta *et al.*, 1965;

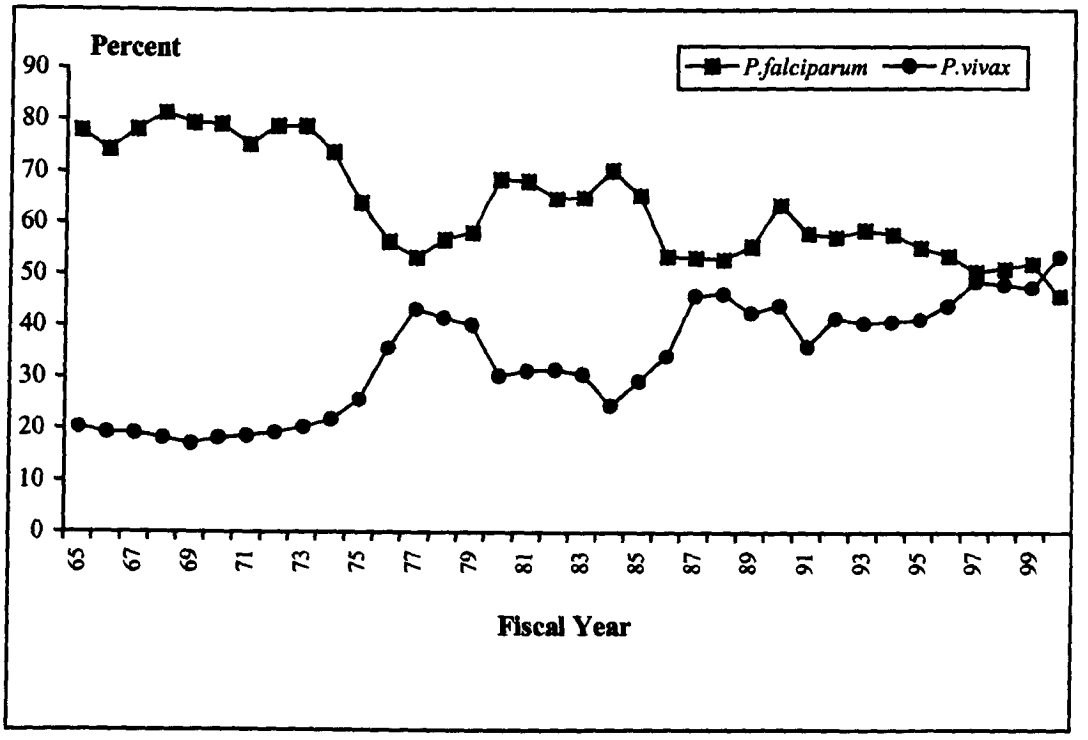
Harinasuta *et al.*, 1967; Bourke *et al.*, 1966; Chin, 1970) (see section 1.5.2 on drug resistance). The adoption of sulfadoxine/pyrimethamine in 1971 (Chin *et al.*, 1966; Harinasuta *et al.*, 1967; Chin and Rattanarithikul, 1973), caused the proportion of *P. falciparum* to decline during 1975-1979. Then it showed an upward trend during 1980-1984 due to the resistance of this parasite to sulfadoxine/pyrimethamine. Following the introduction of mefloquine/ sulfadoxine/ pyrimethamine (MSP) on an operational basis in 1985 a decline of *P. falciparum* was reported (Malaria Division, 1997), and the further decline in the 1990s is presumably associated with the introduction of artesunate and mefloquine as described in section 1.5.

P. vivax is the second most important species in the country, its effect on malaria mortality is minimal, but it has a considerable influence on morbidity because relapse is very common in *vivax* infections.

The human reservoirs which infect mosquitoes with both *P. falciparum* and *P. vivax* were studied in the research described in this thesis.

Though *P. malariae* and *P. ovale* also exist in the country, both are reported to be less than 1% of all infections.

The most recent reports of the Malaria Division (2000) have specified the frequency of *P. falciparum*, *P. vivax*, *P. malariae* and mixed infections as 45.9%, 53.5%, 0.1%, and 0.5% respectively. The year 2000 was the first year in the 50 years for which data are available in which *P. falciparum* infection was less common than *P. vivax*.



Source: Annual report for 1994 to 2000, Malaria Division, Ministry of Public Health

Figure 1.13 Proportion of the two main malaria parasite species in Thailand from 1965-2000.

The proportions of parasite species in northern Thailand in 2001 were approximately 47% *P. falciparum*, 51% *P. vivax*, 0.2% *P. malariae* and 1.2% mixed infections (Office of Vector-borne Diseases Control No. 2, 1997; 2001) (Table 1.5).

Table 1.5 Blood slide examination in northern Thailand from 1996-2001.

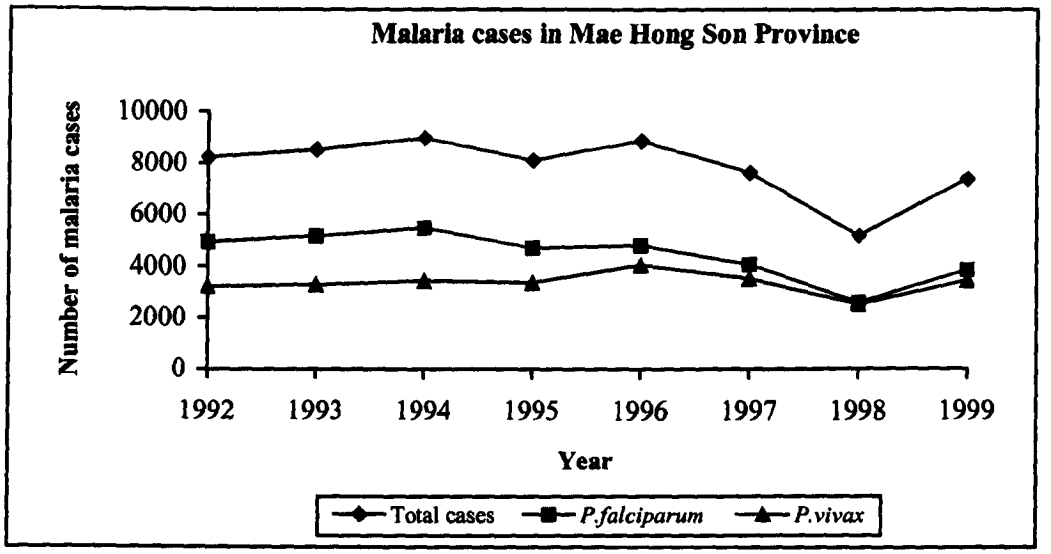
Year	Blood exam.	Cases (%)	Species				
			Pf (%)	Fg [a] (%)	Pv (%)	Pm (%)	Mixed (%)
1996	1,085,123 [b]	17,656 (1.63)	8,572 (48.55)	1,349 (15.74)	8,971 (50.81)	17 (0.01)	96 (0.54)
1997	1,027,452 [b]	18,260 (1.78)	8,731 (47.81)	1,487 (17.03)	9,383 (51.39)	14 (0.08)	132 (0.72)
1998	1,116,743 [b]	12,519 (1.12)	5,264 (42.05)	495 (3.95)	7,151 (57.12)	8 (0.06)	96 (0.77)
1999	1,343,242 [b]	18,404 (1.37)	8,648 (46.99)	861 (4.68)	9,605 (52.19)	26 (0.14)	125 (0.68)
2000	1,323,761 [c]	12,619 (0.95)	5,566 (44.11)	492 (8.84)	6,906 (54.74)	33 (0.25)	114 (0.90)
2001	1,360,625 [c]	10,475 (0.77)	4,966 (47.41)	434 (8.74)	5,355 (51.12)	23 (0.22)	131 (1.25)

[a] falciparum gametocytes, Pf = *P. falciparum*, Pv = *P. vivax*, mixed = mixed infection

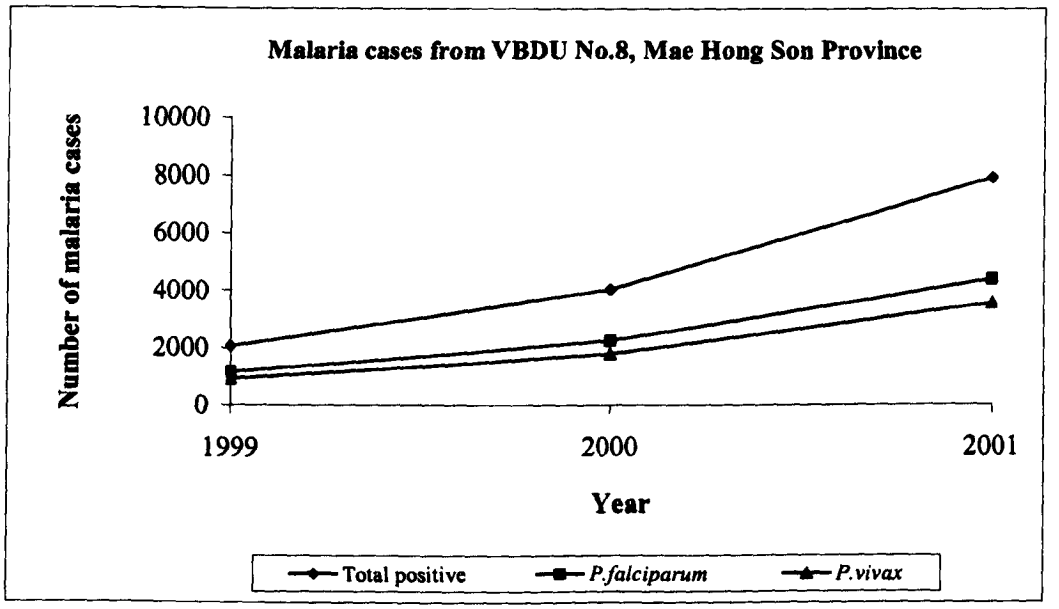
[b] 13 provinces in northern Thailand, ie. Mae Hong Son, Lampang, Lamphun, Phare, Nan, Auttaradit, Pisanulok, Sukotai, Phetchabun, Phichit, Phayao, and Chaing Mai.

[c] Following re-organisation of the area stratification, 12 provinces are included with Sukotai province now included in a different zone.

In Mae Hong Son Province *P. falciparum* is generally slightly more common than *P. vivax*, but in 1999 the frequencies were almost equal. The situation is similar in the area where the present project was carried out. This area is under the responsibility of VBDU No.8 (Figure 1.14). The monthly proportions of these parasites as detected by all types of surveillance activities in this unit is shown in Figure 1.15.

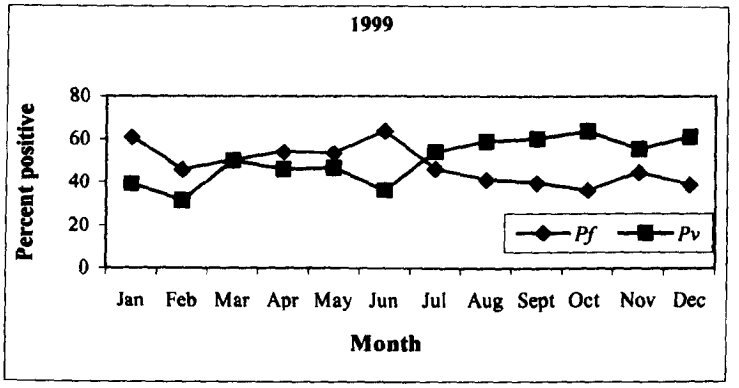


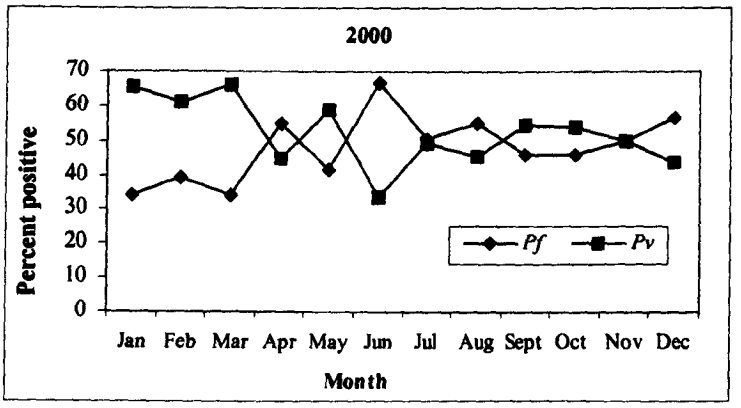
Source: Annual report 1994 to 1999, Malaria Division, Ministry of Public Health

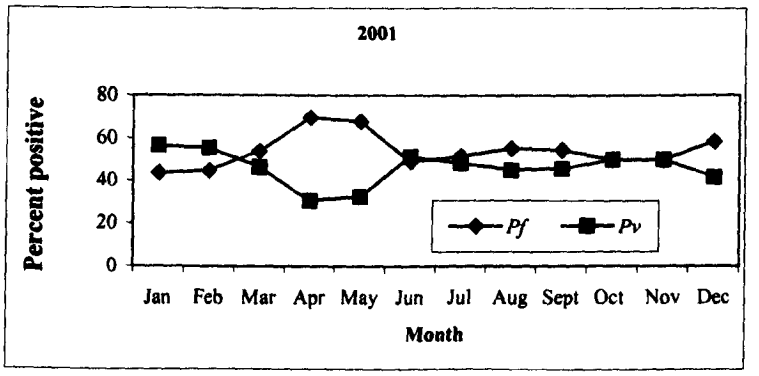


Source: Vector-borne Disease Control Unit no.8, Mae Hong Son Province, Thailand

Figure 1.14 Malaria cases and parasite species among Thai nationals in Mae Hong Son Province, 1992-2000 and from the Vector-borne Disease Control Unit No.8 (only 4 cantons), 1999-2001



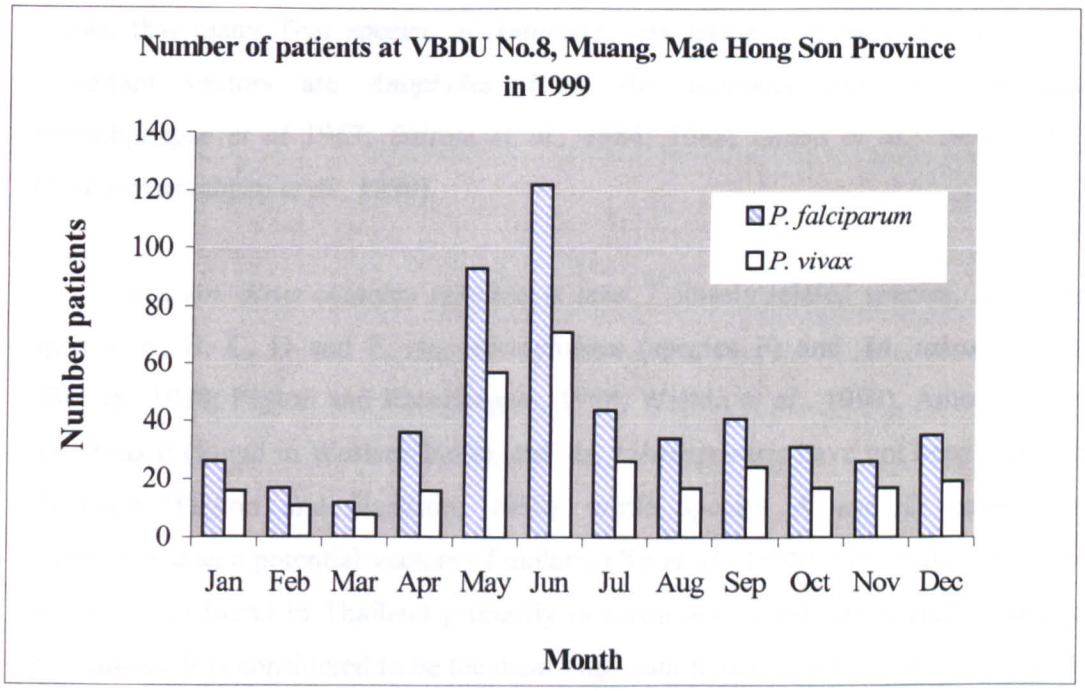




Source: Vector-borne Disease Control, Unit no.8, Mae Hong Son Province, Thailand

Figure 1.15 Percent positive per month for two parasite species from all malaria activities at VBDU No. 8 in 1999-2001.

In 1999, the number of patients who came to the Office of Vector-borne Disease Control Unit No. 8 was highest during the rainy season (May-July) and *P. falciparum* malaria was the dominant species at all seasons (Figure 1.16).



Source: Vector-borne Disease Control Unit no 8 (VBDU), Mae Hong Son Province, Thailand

Figure 1.16 Monthly reported numbers of patients at Vector-borne Disease Control Unit No. 8, Muang district, Mae Hong Son Province (1999), by parasite species

1.4 The Vectors

There are 410 species of mosquitoes in Thailand but only 72 species belong to the genus *Anopheles* (Harrison *et al.*, 1990). Several studies (Kasemsuth *et al.*, 1988; Somboon *et al.*, 1994; Rattanarithikul *et al.*, 1996; Rongsriyam *et al.*, 1998), using dissection or ELISA for detection of sporozoites in salivary glands, have shown that many Thai species of *Anopheles* can transmit malaria. However, the important vectors are *Anopheles dirus*, *An. minimus* and *An. maculatus* (Pinichpongse *et al* 1967; Baimai *et al.*, 1984; 1988; Green *et al.*, 1990; 1992; Chareonviriyahpap *et al.*, 1999).

The *An. dirus* complex contains at least 7 closely related species, *An. dirus* species A, B, C, D and E, *An. nemophilous* (species F) and *An. takasagoensis* (Baimai, 1988; Peyton and Ramalingam, 1988; Walton *et al.*, 1999). Among these, *An. dirus* E (found in Western India), and *An. takasagoensis* have not been found in Thailand (Peyton and Harrison, 1980), while species A and D have been incriminated as a potential vectors of malaria (Xu *et al.*, 1998). Generally, *An. dirus sensu lato* is found in Thailand primarily in forest and forest-fringe areas and tree plantations. It is considered to be the most important malaria vector in forest areas of several provinces in Thailand especially along the mountainous border with Cambodia (Wilkinson, 1978; Rosenberg *et al.*, 1990b), although other species also occur there (Prasittisuk, 1985). Somboon (1993) reported from Northwest Thailand that *An.dirus s.l.* contributed to transmission especially in the early rainy season when their populations were relatively high. Species A of *An. dirus s.l.* is widespread throughout Central and Northeast Thailand, but rare in the Northwest (Somboon, 1993). Species B and C are found in the southern peninsula. Species D is common along the Thai-Myanmar border in the west (Baimai, 1989). *An. dirus s.l.* breeds mainly in small pools, animal footprints or rock pools under shade with dense vegetation (Scanlon and Scandhinand, 1965; Wilkinson, 1978). Because of the association of malaria with *An. dirus s.l.* almost all malaria in Thailand occurs in areas covered either with natural forest, orchards, or tree plantations. *An. dirus s.l.* prefers to bite humans, avoids contact with DDT because it does not rest indoors,

either before or after feeding, and is long-lived (Scanlon and Scandhinand, 1965; Ismail *et al.*, 1974; 1975; 1978). All of these characteristics make it a very efficient vector, in spite of generally low population densities (Rattanarithikul and Panthusiri, 1994). Rosenberg *et al.* (1990a; 1990b) reported from south-eastern Thailand that *An. dirus s.l.* bit outdoors between 2200 and 0100 h and only *An. dirus* was found to be infective. He showed that February was the month of highest transmission (inoculation rate highest) and the number of people showing *P. falciparum* gametocytes in their blood increased by more than 300% in December, or within 30 days of catching the first infective *An. dirus s.l.* in that transmission season. The same author reported that the number of gametocyte carriers remained high throughout the dry months (November to May) but then steeply declined soon after the last infective mosquito was found, about the time that the annual monsoon began.

An. minimus s.l. is widely distributed throughout Thailand except in the provinces in the south along the border with Malaysia. This species is commonly found along the shaded, forested and cleared forested foothill areas with slowly moving streams, except in the north where it is also found in irrigation canals for rice paddies close to mountains. Vector contact with humans is usually greatest along the margin of villages. *An. minimus s.l.* bites early in the night during the cool dry season, but throughout the night in the wet season, with peaks before midnight and dawn (Ismail *et al.*, 1974; 1975; 1978; Ratanatham *et al.*, 1988). Adults feed mainly on humans, but also on domestic animals. They mainly feed and rest indoors. This mosquito is often found in association with *An. maculatus*, which breeds in puddles, especially in southern and northern Thailand. The *An. minimus* group consists of 3 related species (Yu, 1987; Sucharit *et al.*, 1988; Baimai, 1989; Green *et al.*, 1990). *An. minimus* species A is commonly found throughout the country, whereas, species C and D are prevalent along the western Thai-Myanmar border, especially in the northwest such as Kanchanaburi and Tak Provinces (Baimai, 1989; Somboon, 1993). Species A was used as the experimental material for the research described in this thesis.

An. maculatus s.l. is a major vector of malaria in peninsular Malaysia (Reid, 1968) and has been recognized as a vector species in southern areas of Thailand close to Malaysia (Scanlon *et al.*, 1968). The *An. maculatus* complex consists of at least 8 closely related sibling species (Kittayapong *et al.*, 1993). *An. maculatus* species E may serve as an important malaria vector in southern Thailand (Baimai, 1989). *An. maculatus* species B also occurs in peninsular Thailand and Malaysia, where it plays a major role in transmission of human malaria (Baimai *et al.*, 1988). The breeding sites of *An. maculatus s.l.* are mainly in streams or rock pools. Adults bite domestic animals and also humans indoors and outdoors, and rest mainly outdoors after feeding.

Other *Anopheles* species such as *An. sundaicus* (coastal, mangrove wetland zones) (Ketrangsee *et al.*, 1991), *An. aconitus* and *An. philippinensis* (both associated with inland, rice field habitats) (Green *et al.*, 1991) are considered to be secondary vectors in some areas (Prasittisuk, 1985; Harinasuta, 1987).

All these species of *Anopheles* have well-defined behavioural characteristics and these often determine the distribution of malaria. As mentioned above in describing the behaviour of the main vectors, it is important to consider their biting behaviour in relation to the behaviour of their human bloodmeal hosts who are mostly rice farmers. In the planting season, farmers wake up at dawn (when is the biting peak of *An. minimus* takes place in the wet season) and they work from dawn to dusk. Some fields are very far from their houses so they need to stay in the fields overnight. In the fields they sleep in huts with one open end and most of them sleep without nets. While sleeping in these huts they may contact *An. dirus* which prefers to bite and rest outdoors. In addition to behavioural and climatic conditions, other factors affecting the breeding, feeding and survival of *Anopheles* must be taken into consideration in any control programme based on anti-mosquito measures.

1.5 Anti-malarial drugs and drug resistance

1.5.1 Anti-malarial drugs in Thailand

Drug regimens used in Thailand are different for each species of *Plasmodium*. All microscopically confirmed *P. falciparum* cases are treated according to the level of mefloquine resistance in the area (Table 1.6). *P. vivax*, *P. ovale* and *P. malariae* infections are treated as shown in Table 1.7. Health centres and malaria health volunteers give presumptive treatment using sulfadoxine/pyrimethamine (1000/50 mg) together with 30mg primaquine, to symptomatic people in villages. Realising that presumptive treatment is at too low a dose for elimination of parasites and may induce drug resistance, the Control Programme decided to phase out such treatment by the end of 2001. Early diagnosis and prompt radical treatment according to parasite species is promoted to replace presumptive treatment.

In the present study, all patients were given anti-malarial drugs according to the regimens as mentioned in Tables 1.6 (low mefloquine resistance) & 1.7.

Table 1.6 Drug regimens for uncomplicated *P. falciparum* treatment in Thailand.

Treatment	Areas categorised by mefloquine resistance		
	Low	**Moderate**	**High**
1st line drug	M 750 mg and P 30 mg, single dose	Day 1: M 750 mg +A 300 mg, single dose in front of a malaria staff member Day 2: A 300 mg for a first meal and P 30 mg for second meal, 6 hours interval	Day 1: M 750 mg+ A 300 mg (first meal) and M 500 mg (second meal). Day 2: A 300 mg for a first meal and P 30 mg for second meal, 6 hours interval
2nd line drug*	Q 600 mg 3 doses with T 500 mg 2 doses, for 7 days	Q 600 mg 3 doses with T 500 mg 2 doses, for 7 days	Q 600 mg 3 doses with T 500 mg 2 doses, for 7 days
3rd line drug**	Day 1 A 300 mg for first meal and P 30 mg for the second meal Day 2-5 A 100 mg once a day	Day 1 A 300 mg (first meal) and P 30 mg (second meal) Day 2-5 A 100 mg once a day	Day 1 A 300 mg (first meal) and P 30 mg (second meal) Day 2-5 A 100 mg once a day

Source: Annual Report 2000, Malaria Division, Ministry of Public Health.

M =Mefloquine (250 mg per tablet), Q =Quinine (300 mg per tablet), P =primaquine (15 mg per tablet), T =Tetracycline (250 mg per tablet), A = Artesunate or artemether (50 mg per tablet)

* Used if parasites are found at day 28 after treatment with 1st line drug

** Used if parasites are found at day 28 after treatment with 2nd line drug

Table 1.7 Drug regimens for *P. vivax*, *P. ovale* and *P. malariae* treatment in Thailand.

Days	*P. vivax*	*P. ovale*	*P. malariae*
1	C 300 mg is given alone for the first and second dose and with P 15 mg for the third dose.	Same as *P. vivax*	Same as *P. vivax* but P is not given
2-3	C 300 mg + P 15 mg, one dose per day	Same as *P. vivax*	Same as *P. vivax* but P is not given
4-14	P15 mg daily	Same as *P. vivax*	Same as *P. vivax* but P is not given

Source: Annual Report 2000, Malaria Division, Ministry of Public Health.

C = Chloroquine (150 mg per tablet), P = Primaquine (15 mg per tablet)

1.5.2 Drug resistance

Drug resistant malaria has been described as one of the most important current problems in tropical medicine, especially in *falciparum* malaria (Fevre *et al.*, 1999).

P. vivax strains resistant to anti-malarial drugs have emerged over the last decade. The resistance of this species to chloroquine (Baird *et al.*, 1997a; 1997b), pyrimethamine (de Pecoulas *et al.*, 1998), and primaquine (Collins and Jeffery, 1996) has been reported in several parts of the world. For unknown reasons there is no problem with drug resistance in *P. vivax* in Thailand and chloroquine-primaquine is still very effective.

Drug resistance is closely related to mass population movements, inadequate health services, improper use of antimalarial drugs, limited resources and operational difficulties in implementing malaria control (Fungladda and Butraporn, 1992; Kidson, 1993; Singhasivanon, 1999). In Thailand, since the first discovery of the resistance of *P. falciparum* to chloroquine by Harinasuta *et al.* (1962), several

studies have reported that *P. falciparum* is resistant to this drug (Harinasuta *et al.*, 1965; Kain *et al.*, 1994) and other drugs such as mefloquine (Karwacki *et al.*, 1989; Nosten *et al.*, 1991; ter Kuile *et al.*, 1992; Wongsrichanalai *et al.*, 1992; Karbwang *et al.*, 1993; Thimasarn *et al.*, 1995), especially on the Thai-Cambodian border.

In 1973, sulfadoxine/pyrimethamine (Fansidar ^{R} or SP) was first officially introduced. In 1981, the control programme was forced to increase the adult dosage of SP from 2 to 3 tablets, and quinine for 3 days plus tetracycline for 7 days was administered as the second line drug. The presumptive treatment was switched to 2 tablets of SP in areas considered as "highly resistant" whilst chloroquine continued to be used in areas considered as "low and medium resistant". SP was widely used as chemoprophylaxis and was sold in private drug stores without control so the resistance to this drug increased very quickly. In 1983, since the spread of resistant strains had been very rapid, the control programme replaced SP as first line drug with quinine/tetracycline in some areas. During 1983-1984, Suebsaeng *et al.* (1986) reported a reduction in sensitivity to quinine and mefloquine. These observations played a major role in the decision by the programme to introduce mefloquine/ sulfadoxine/pyrimethamine (MSP or Fansimef) as an alternative treatment. Following its introduction on an operational basis in 1985 a further decrease in sensitivity to both quinine and mefloquine has been noted.

Because of the administrative difficulties arising from having different regimens in different areas, stratified according to drug resistance level, it was decided to adopt a standard regimen for *P. falciparum* according to mefloquine resistance (detailed in Table 1.6).

As described previously, from 1988 to 1991 Thailand experienced an epidemic of *P. falciparum* among gem miners returning from Cambodia. The epidemic was centred in Bo Rai district, Trat province, eastern Thailand, close to the Pailin gem mine in Cambodia (Thimasarn *et al.*, 1990). This area is well known as the epicentre of multidrug resistance. Many miners died and others carried drug resistant malaria with them, largely to Trat (east) and Tak (west) Provinces. There

was a rapid rise in resistant strains in those areas, as indicated by a precipitous fall in MSP (Mefloquine S-P) cure rate over a short time frame in the two provinces. From Tak the resistant strains rapidly spread to Myanmar and beyond. This multi-drug resistance spread to other areas of the country, such as the Thai-Myanmar border (Nosten *et al.*, 1991), and other places on the Thai-Cambodian border (Johnson *et al.*, 1982; Thimasarn, 1992). A report of the Malaria Division (1991) showed that MSP treatment failure was as high as 70% in the Eastern Provinces. In some areas the treatment failure rates in children with acute *falciparum* malaria after administration of high-dose mefloquine, exceeded 50% (Nosten *et al.*, 1991; Price *et al.*, 1996).

Because of resistance of *P. falciparum* to chloroquine, sulfadoxine-pyrimethamine, quinine and mefloquine, a new anti-malarial drug or a combination with the existing drugs was considered necessary to provide a way in which resistance could be combated. The use of *Artemesia* derivatives, alone or in combination with mefloquine, has been shown to be effective against multidrug resistant *P. falciparum* in many clinical trials (e.g. Bunnag *et al.*, 1991a; 1991b; White *et al.*, 1992; Nosten *et al.*, 1998; 2000).

Price *et al* (1996) showed that when artemisinin derivatives were introduced as a component of first-line treatment, there was a significant reduction in the incidence of clinical *P. falciparum* malaria. In 1998 he showed excellent results with a combination of artesunate and mefloquine in the treatment of uncomplicated multidrug-resistant *P. falciparum*. He found that the failure rate in those receiving artesunate plus mefloquine was 7%, compared with 26% in patients treated with artesunate alone. Thimasarn *et al* (1997) showed excellent results of artesunate 600 mg or artemether 640 mg in combination with mefloquine 1,250 mg over a period of two days. She also concluded that these drugs should be considered for alternative regimens for treating uncomplicated multi-drug resistant *falciparum* malaria.

Chlorproguanil/dapsone (LapDap) gave good results in Kenya and Tanzania in eliminating *P. falciparum* infection (Watkins *et al.*, 1997; Maxwell *et al.*, 1999). This result contrasts with a low effectiveness of LapDap in Thailand (Wilairatana *et al.*, 1997) as well as in other parts of South-East Asia because of the presence of resistance genes, especially the leu 164 allele of DHFR (dihydrofolate reductase) (Watkins *et al.*, 1997). This gene is a mutation of DHFR. It is believed that leu 164 is the cause of failure of chlorproguanil with dapsone (CP-DS) in south-East Asia (Watkins *et al.*, 1997). Mutabingwa *et al.* (2001) showed that the leu 164 mutant was found in several samples obtained from Laos but none from Tanzania.

Because of the threat of further drug resistance, it is necessary to study the pharmaco-kinetics, as well as side effects, of new anti-malarial drugs. There are several drugs that are in various stages of development that may well have a role in malaria control and treatment. These include pyronaridine, tafenoquine, and bulaquine.

1.6 Studies on anti-gametocyte activity of drugs

The rate of malaria transmission and the spread of resistance could be reduced if the drug(s) used have an effect on gametocytes (the sexual stage of the malaria parasite responsible for infection of mosquitoes). Numerous studies have been reported on the activity against gametocytes of certain anti-malarials such as primaquine (Bunnag *et al.*, 1980; Chomcharn *et al.*, 1980; Doi *et al.*, 1989; Kaneko *et al.*, 1989; Kazazian *et al.*, 1992; Pukrittayakamee *et al.*, 1994; Singhasivanon *et al.*, 1994), pyronaridine (Chavalitshewinkoon-Petmitr *et al.*, 2000) and artemisinin (Chen *et al.*, 1994; Doherty *et al.*, 1999; von Seidlein *et al.*, 2000).

The research described in this thesis has bearing on the identification of which section(s) of the population would have to be treated with an anti-gametocyte drug to reduce the reservoir from which wild mosquitoes are infected.

1.6.1 Primaquine

Primaquine is the best known gametocytocidal drug. This drug eliminates the infectivity of gametocytes of *P. falciparum* within 6-12 hours of administration (Jeffery *et al.*, 1956). Many authors (Barber *et al.*, 1929; Mackerras and Ercole, 1949; Burgess and Bray, 1961; Rieckmann *et al.*, 1968; 1969; Strickland *et al.*, 1986) have noted the rapid action of this drug in suppressing infectiousness of *P. falciparum* in humans. This drug has long been used for prevention of relapses of malaria, and in recent decades, it has been re-examined for use in malaria prevention by eliminating infections in the liver.

However, there are some potential side effects of this drug such as gastro-intestinal toxicity, and acute haemolysis. Primaquine-induced gastro-intestinal disturbances can be minimised if the drug is taken with food. The most serious side effect of this drug is acute haemolysis in people with G6PD (Glucose-6-phosphate dehydrogenase) deficiency (Carson *et al.*, 1981). Bangchang *et al.* (1994) showed that haemolysis varied in severity but was not related to the degree of G6PD deficiency. There was no difference in the plasma concentration or pharmacokinetics of primaquine between Thai male patients with G6PD and non-G6PD deficiency infected with *P. vivax*. However, it is very important to check the G6PD deficiency status of patients before giving this drug in Thailand.

Several studies of the action of this drug against sexual stages of *Plasmodium* have been reported. Chomcharn *et al.* (1980) showed that a single dose of 45 mg of primaquine base was effective in the elimination of gametocytes of chloroquine-resistant *P. falciparum* in Thailand. The gametocyte counts decreased markedly within 3.5 days, showing a significant difference from the controls, in most of whom gametocytes still remained for 11 days. He suggested that primaquine can be used as a gametocytocide and sporontocide for *falciparum* malaria in Thailand if the drug was administered when mature gametocytes appeared in the peripheral blood. Singhasivanon *et al* (1994) stated that the co-administration of MSP and primaquine

in children would be beneficial by reducing the transmission of malaria in endemic areas.

Kaneko *et al.* (1989) investigated the effect of primaquine as a gametocytocidal drug in 218 *P. falciparum* malaria cases detected during passive case detection (PCD) in two coastal villages of North Sumatra, Indonesia, where chloroquine-resistant and Fansidar-sensitive *P. falciparum* were prevalent. He found that the parasite rate declined after PCD activities and also the gametocyte positive rate (GPR) was reduced from 77% on day 0 to 7% at week 3. He indicated that a single dose of 45 mg primaquine with sulfonamide + pyrimethamine (SP) was partially effective in reducing gametocytes and reducing the malaria prevalence rate when administered after PCD.

There is an analogue for primaquine called tafenoquine (WR 238605). Numerous studies have been reported about this drug both in animals (Anders *et al.*, 1988) and humans (Kazazian *et al.*, 1992; Cooper *et al.*, 1994; Brueckner *et al.*, 1998a; 1998b; Peters, 1999; Lell *et al.*, 2000; Nicolas *et al.*, 2001; Shanks *et al.*, 2001). Pre-clinical studies demonstrated that it has greater efficacy and less toxicity compared with primaquine and it also has a long absorption phase and is slowly metabolized (Brueckner *et al.*, 1998a). It has a long half-life, is well tolerated and active against liver-stage malaria parasites (Lell *et al.*, 2000; Shanks *et al.*, 2001). It was planned that advanced field testing of this drug would begin at the end of 2001 and the further study of its action against the gametocyte stage is needed.

1.6.2 Artemesia derivatives

Artemisinin has high efficacy against asexual *falciparum* and *vivax* malaria but has no anti-hypnozoite activity. Several studies have been reported on the effect of this drug on the gametocyte stage both using this drug alone or combined with the other anti-malarial drugs. Dutta *et al.* (1989) first commented on the possible use of this drug to stop malaria transmission. Gametocytocidal activity has been demonstrated against *P. cynomolgi* (simian malaria parasite). A single intramuscular

injection of 5 mg/kg artemisinin on gametocyte-carrying monkeys resulted in complete loss of mosquito infectivity within 24 hr.

Chen *et al.* (1994) reported from China that the density of gametocytes and infectivity to *An. dirus* were significantly reduced on days 4, 7, 14 and 21 after treatment with a daily dose of 1200 mg artemisinin. This indicated that artemisinin can effectively influence the infectivity of gametocytes of *P. falciparum.*

Studies in the Gambia showed markedly lower gametocyte prevalence after treatment with the combination of artesunate and pyrimethamine-sulfadoxine than after treatment with pyrimethamine-sulfadoxine alone (Doherty *et al.*, 1999; von Seidlein *et al.*, 2000). These authors indicated that the combined treatment of artesunate with pyrimethamine-sulphadoxine was safe, well tolerated, and effective. However, it remains unclear from these studies whether the observed reduction in gametocyte numbers is due to the gametocytocidal effect of artemisinin, to the lower production of gametocytes due to rapid reduction in the total parasite burden, or to a combination of both.

Targett *et al.* (2001) determined the infectivity to mosquitoes of gametocytes in peripheral blood after three possible treatments (chloroquine alone, pyrimethamine-sulfadoxine alone, or artesunate with pyrimethamine-sulfadoxine) by using membrane feeding. Infection of mosquitoes was observed in all groups but it was lowest in those who received pyrimethamine-sulfadoxine and 3 doses of artesunate.

1.7 Mosquito feeding and the development of *Plasmodium* in mosquitoes

The estimation of the infectiousness of the *Plasmodium* reservoir to mosquitoes helps in understanding the epidemiology of malaria and its changes after application of control measures. There are several ways to estimate this

infectiousness. A direct approach is to feed batches of mosquitoes on a demographically representative human population, regardless of parasitaemia or gametocytaemia, either by membrane feeding or by feeding laboratory-bred mosquitoes directly on the skin of individuals. The earliest studies were from the 1930s to early 50s when malaria therapy was used in the treatment of neurosyphilis. Measurement of the sporozoite rates in wild anophelines in relation to ovarian age grade, which is proportional to the number blood meals that each mosquito has taken, is another approach for measurement of the infectiousness of the human population to mosquitoes (Lines *et al.*, 1991).

Different approaches have been used for the above purposes including direct feeding on the skin of individuals (Muirhead-Thomson, 1954; Graves *et al.*, 1988; Gamage Mendis *et al.*, 1991; Sattabongkot *et al.*, 1991; Githeko *et al.*, 1992), or feeding through an artificial membrane (Rutledge 1964; Graves, 1980; Ponnudurai *et al.*, 1989; Boudin *et al.*, 1991; 1993; Tchuinkam *et al.*, 1993; Robert *et al.*, 1996; Gouagna *et al.*, 1998). Many investigators have compared the two approaches. Collins *et al.* (1964) and Vanderberg (1980) showed that the infection rates in mosquitoes fed through membranes usually equalled or exceeded infections in mosquitoes fed directly on animals or humans. More recently Bonnet *et al.* (2000) reviewed the results of comparisons of artificial membrane feeding with direct feeding in *An. gambiae*. They concluded that both techniques can be used to evaluate human infectiousness to mosquitoes but this analysis in contrast to those of Collins *et al.* (1964) and Vanderberg (1980), showed that direct feeding gave better results in terms of infection than membrane feeding. A very recent study by Awono Ambene *et al.* (2001) showed that the infection of *An. arabiensis* with *P. falciparum* by membrane feeding was similar to infection by direct feeding.

Table 1.8 shows the results from several studies that have been conducted to investigate the infectivity of *Plasmodium* species to various species of *Anopheles* and also includes the comparative studies of direct and membrane feeding methods.

The infection rates varied in each study. The differences could be explained in three ways.

1. Different sources of human malaria infections; symptomatic gametocyte carriers, asymptomatic carriers or carriers from a demographically representative human population were used in different studies. A few studies from areas of intense seasonal transmission in Africa showed that adults were more infectious to mosquitoes than children although gametocytaemia was higher in children (Drakeley *et al.*, 2000). This may be explained in terms of a crowding effect, whereby the higher gametocyte densities in the younger age groups led to intense competition and lower rates of successful infection. Other relevant factors could be the effect of transmission blocking or enhancing antibodies or of parasite-killing disease-inducing factors. Lensen *et al.* (1998) conducted membrane feeding on gametocyte carriers from Cameroon and travellers who had had a first malaria experience. The results showed that the infectivity of gametocytes from semi-immune carriers was significantly lower compared with that of non-immune carriers. The immunity involved must be directed against the gametocytes inside the human and is distinct from artificially induced transmission-blocking immunity, which would act in the mosquito midgut, affecting the extra-erythrocytic gamete and/or zygote stages of the parasite.

2. The quantity and quality of gametocytes may play an important role on mosquito infection. Many investigators have shown that very low or undetectable densities of gametocytes could infect mosquitoes. There appeared to be a weak correlation between the number of mosquitoes infected and gametocyte density (Jeffery, 1952; Muirhead-Thomson, 1954; Jeffery and Eyles, 1955; Graves *et al.*, 1988; Sattabongkot *et al.*, 1991; Haji *et al.*, 1996; Price *et al.*, 1996; Bonnet *et al.*, 2000). However, different studies have produced conflicting results on this subject

(Boyd and Kitchen, 1937; Muirhead-Thomson, 1957; Constantinescu and Negulici, 1967; Carter, and Gwadz, 1980; Graves, 1980; Boudin *et al.*, 1993). Some cases with high gametocyte densities could not infect mosquitoes and increase toxins from parasites may play an important role in infectivity. Naotunne *et al.* (1993) showed that the infectivity of gametocytes of infected mice after being fed on by *An. stephensi*, was reduced during schizogony in the blood. This may be caused by the blocking of tumour necrosis factor (TNF) by malaria antigen. One in vitro study also showed that the serum from *P. vivax* semi-immune individuals blocks the induction of TNF and parasite-killing factors by parasite antigen. Thus the reduction of infectivity of *P. vivax* and *P. yoelii*-infected gametocyte carriers to mosquitoes was observed (Motard, 1990; Naotunne *et al.*, 1991; Karunaweera *et al.*, 1992). Sinden (1991) showed that infectivity of gametocytes to mosquitoes increased when parasitaemia decreased.

3. *Anopheles* mosquitoes vary in their vector potential because of environmental conditions and factors affecting their abundance, blood-feeding behaviour, survival, and ability to support malaria parasite development. In the mosquito, the three main phases at which parasite development may be interrupted are at the developmental transitions between gametocyte and ookinete stages, between ookinetes and mature oocysts and between oocysts and sporozoites in the salivary glands. For the ookinete to oocyst transition, two mechanisms that inhibit development include failure of ookinetes to traverse the midgut and abortion of early-stage oocysts. Parasite development in the mosquito midgut progresses in association with processes of mosquito blood-meal digestion. Larger mosquitoes take a larger blood meal, resulting in a higher level of infection (Kitthawee *et al.*, 1992; Kelly and Edman, 1992; Lyimo and Koella, 1992). The mosquito's age may also affect infection, the survival rate until sporozoite maturity being higher in younger than the older mosquitoes. The number of bloodmeals taken may also influence the probability of infecting the mosquito.

Table 1.8 Summary of data on using direct or membrane feeding.

Year	Authors	Where	Objectives	Methods/subjects	Comment
1937	Boyd and Kitchen, 1937	Florida	To study the infectiousness of patients infected with *P. vivax* or *P. falciparum*	• Direct feeding • 10 female *An. quadrimaculatus* were fed daily on patients starting from the first discovery of parasites. • 415 patients, who were each inoculated by seven *An. quadrimaculatus* infected with *P. vivax*, and 1073 African American patients who were each inoculated with 11 *An. quadrimaculatus* infected with *P. falciparum*	***P. vivax*** • Parasites were detected 14 days after inoculation • The temperature rose 14 days after inoculation. • The gametocytes were first observed on the sixteenth day and the last detection on the twenty-sixth day. • The patient was infectious to mosquitoes with densities of less than 10 gametocytes per cubic millimeter. • The mosquitoes applied 15 days after inoculation were the first to become infected, and they continued to become infected as long as they were applied, even after the gametocyte densities were undetectable.

	Boyd and Kitchen (cont.)				***P. falciparum*** • Parasites were first recovered 14 days after inoculation, the same day as the first rise in temperature. • Gametocytes were first detected on the twenty-third day following inoculation or 10 days after the first appearance of parasites, and continuously present for 27 days. • The patient was not infectious until late in the clinical attack. None of the mosquitoes applied during the first appearance of gametocytes became infected. • No infection was secured with undetectable densities of gametocytes or densities less than 10 per cmm.

1952	Jeffery, 1952	USA	To describe events during early primary parasitemias	• Direct feeding • 100 *An. quadrimaculatus*, daily feeding, mean of 34.5 mosquitoes per batch were dissected. • 24 Neurosyphilitic patients were inoculated by the bites of infected mosquitoes with the Chesson strain of *P. vivax*.	• Parasites appeared about 12 days after inoculation and gametocytes, on an average, about six days later • First infected mosquitoes were obtained on an average about 16 days after inoculation of the patient or on the fourth day of detected parasitaemia. • In one case, the mosquito infections appeared before gametocytes were demonstrable by routine blood examinations. • The onset of fever occurred, on an average, two days before first infection of mosquitoes and two days following demonstrable parasites. • In 4 cases the first onset of fever (100°F) appeared on the same day that mosquitoes were first infected.

1955	Jeffery and Eyles, 1955	Milledgeville, Georgia, State, USA	To test the duration of *P.falciparum* infections in humans of the South Carolina and Panama strains of *P. falciparum*, as related to gametocyte level and stage of infection.	• Direct feeding • *An. quadrimaculatus* was used for South Carolina strain and *An.albimanus* was used in case of Panama strain. • *P. falciparum* • 27.5 mosquitoes per batch were dissected on 12 ± 1 days after the blood meal. • Patients were neurosyphilitic African Americans, inoculated either by sporozoites or by intravenous injection. 29 cases were with South Carolina and 59 cases with Panama strain.	• infectivity to mosquitoes of observed gametocyte densities of 0-100 per cmm was found. • The number of failures encountered in batches fed when the gametocyte densities were between 100 and 1000 per cmm, indicating that gametocyte densities do not invariably guarantee good mosquito infections. • The infections were of lesser intensity and more irregularly seen when gametocyte densities were low and when the infection had endured for a long time.

1957	Muirhead-Thomson, 1957	West Africa	To determine the proportion of persons in a malarious area who are infectious to mosquitoes.	• Direct feeding • *An. gambiae* • All individuals of *P.falciparum*	• 10-11% of all individuals were infectious to mosquitoes • The infectivity rates of children were significantly higher than in adults • He proposed that children represented the major infectious reservoir of malaria parasites.
1980	Graves, 1980	The Gambia	To investigate the optimum feeding time required to produce reliable infections. To investigate the relationship between gametocytemia and infectivity to mosquitoes.	• membrane feeding • 4-6 day old mosquitoes of the Fajara strain of *An. gambiae s.s.* • *P. falciparum* gametocyte carriers, mostly children under five.	• For maximum infections, mosquito feeding should be completed within 15 minutes, or less, of the blood leaving the patients. This agrees with the study of Sinden *et al* (1978) who showed that gametocyte activation and exflagellation in *P.falciparum* are completed within 30 minutes in vitro at 29° C. • The lowest gametocytemia which produced an infection, was 56/mm^3, but a lower limit of 300 gametocytes/mm^3 was found to be useful for selecting donors.

1988	Graves *et al.*, 1988	Madang, Papua New Guinea	To study the malarial infectiousness of human populations to mosquitoes.	• 20-30 *An. farauti* • Both parasite species • In 1984 membrane feeding was used, but in 1985, direct feeding was used (because of the reluctance of mosquitoes to feed through membranes) on a demographically representative human population. • Infectivity was calculated based on determining the prevalence of gametocyte carriers and measuring the probability of infection to mosquitoes from a known gametocyte carrier.	<u>Feeding on the village population</u> • Only 4% (8/201) individuals were infectious to mosquitoes, among those 7 cases were aged <20 years, and the mean percentage from these 8 infectious people was 37.9% • The observable gametocyte rate was 8.5 (17 cases), and 6 of the gametocyte carriers (46%) were infectious. • 2 out of 13 persons (15.4%) had *P. vivax*. Only asexual stages were infectious to mosquitoes, indicating that in these cases *P. vivax* gametocytes had been undetected. This finding was not repeated with *P. falciparum*. • Many young children (0-4 age group) refused to allow mosquitoes to feed on them.

1990	Gamage Mendis *et al.*, 1991	Sri Lanka	To investigate the reservoir of infection at low endemicity	• Direct feeding on *P. vivax* and *P. falciparum* patients at the time they presented for treatment. • 3-4 day-old *An. tessellatus* were used in this study.	• gametocyte rates increase, rather than decrease, with age • the infectivity to mosquitoes is higher in adolescents and adults than in young children. These contrast with the situation described in areas of hyperendemic malaria such as West Africa. • 25% of cases that were gametocyte-negative for *P. vivax* were infectious to mosquitoes. • low number of oocysts per mosquito (<10)

1990	Somboon and Morakote, 1990	Chiang Mai, Thailand	To determine how long malaria gametocytes continue to be able to infect mosquitoes after storage in vitro.	• Membrane feeding using glass feeders warmed with a circulating water pump (Rutledge, 1964). • *P. falciparum* & *P. vivax* • Malaria patients admitted at Chiang Mai hospital	• The maximum infectivity for both species was on the day the blood was collected. • *P.falciparum* kept at 25°C or 37°C, was still infective to mosquitoes up to day 3, but infectivity much higher when the samples were kept at 37°C. • *P. vivax* completely lost its infectivity after one day's storage, regardless of the temperature.

1991	Sattabongkot *et al.*, 1991	Kanchanaburi, Thailand	To examine the infectiousness of 496 males suffering from uncomplicated symptomatic *P. vivax*	• Direct feeding • 200 female *An. dirus* (Bangkok colony) • Patients age >20, male	• More than 55% of subjects had gametocytaemia ≤20 /100 fields and mean density was 26.8/100 fields. • No correlation between the number of mosquitoes infected and gametocyte density and also between gametocyte density and oocyst number. • Mean oocyst number was 9.3 (range 0-141.9). • Trophozoite density was positively correlated to gametocyte density, this finding contrasted with that of Robert (2000). • More than one third of the cases failed to infect any mosquitoes. • 13 cases out of 36 without observable gametocytes could infect mosquitoes.

1992	Githeko *et al.*, 1992	Western Kenya	To investigate the infectious to mosquitoes in all age groups.	• Direct feeding • Subjects: all age groups, both sexes, regardless of parasitaemia and gametocytaemia. • 100 colony-reared *An. gambiae* females were fed on each volunteer.	• Asexual parasitaemia was very high in young children but gametocytemia was not correspondingly high. • 43% in the 1 to 4 year age group were infectious to mosquitoes, and only 20% were found in adults. • level of infection (oocyst density) decreased with age. • Only 3-7% of mosquitoes became infected.

1993	Boudin *et al.*, 1993	Burkina Faso	To estimate the proportion of infectious individuals.	• membrane feeding • 200 *An. gambiae s.s.* per batch, only batches with more than 14 surviving mosquitoes were dissected. 336 mosquitoes survived from control groups fed on calf blood. • Demographically representative human population. 107 families (15% of the population) selected from those volunteering; 322 subjects with 120 children (5-14), 113 adolescents and young adults (15-29) and 89 adults (>29 years old).	• The mean percentage of infected mosquitoes was 11.5%. No significant difference among age groups. There were only 1.89 oocysts per infected mosquito. • Estimated that approx. 40 ± 8.4% of the population above four years of age was infectious to mosquitoes. • The high gametocyte rate, associated with a high infectiousness, was presumably responsible for a high transmission level. • approximately the same proportion of infectious individuals among the gametocyte carriers as among non-gametocyte carriers.

1998	Touré *et al.*, 1998	Bancoumana, Mali	To measure infectivity of gametocyte carriers and compare two assay methods	- Direct feeding, of batches of 30 *An.gambiae s.l.* and membrane feeding of *An. freeborni*. Eight days after the blood meal, approx. 10 from each cage were dissected and examined for oocysts. - Gametocytaemic volunteers age≥ 2 years	• Age group 10-18 years gave the highest infection rate (51.5%). • No correlation of intensity of infection or percent of mosquitoes infected between the direct feed and membrane-feeding assays. However, there was difference in number of oocysts per mosquito i.e. ≤ 6 oocysts from direct feeding and geometric mean of 30 oocysts from membrane feeding assay. • This study used a different mosquito species for the two feeding methods and this may have caused the difference in results.

2000	Pichon *et al.*, 2000	Dakar, Senegal	To investigate the distribution of *P. falciparum* gametocytes into the vector blood meal	• 30-40 female *An. arabiensis* • 3 adult male volunteers naturally infected by *P. falciparum*, carrying gametocytes and asymptomatic. • Volunteer A had been treated with halofantrine, B with chloroquine, and C not treated.	• Volunteers A, B and C harboured mean gametocytaemias of 0.6, 2.9 and 45.7/µl, respectively. • This is the first study that highlights the passage of *P. falciparum* gametocytes from man to mosquito.

2000	Robert *et al.*, 2000	Dakar, Senegal	To investigate the effect of antimalarials on the gametocyte stage in humans and its infectivity for mosquitoes.	• Membrane feeding • 60 females *An. arabiensis* (3-4 days old) were starved for 5-7 hr. • 127 *P. falciparum* infections had been treated with CQ or sulfadoxine plus pyrimethamine • Patients with parasitaemia >2,000/µl and a temperature ≥38°C	• Post-therapeutic gametocytaemia was higher in patients treated with SP than in those treated with CQ. • Overall, 14.1% were infectious at day 0, and 38.5% at day 7. • The mean percentages of infected mosquitoes were 3.2% at day 0 and 12.6% at day 7.

2000	Bonnet *et al.*, 2000	Mengang, Cameroon	To compare mosquito infection rates obtained by membrane and direct feeding on the same individuals.	• *An. gambiae*, only experiments with more than 20 surviving mosquitoes at day 7 were included and analysed. • Direct and membrane feeding • Both observable gametocyte carriers (30 cases) and those who were apparently non-gametocyte carriers (7 cases; as a control) were used. • all age groups	• 1 non-gametocyte carrier could infect mosquitoes • 10 gametocyte carriers failed to infect mosquitoes, 17 gave infection with both tests, 2 gave infection only after direct feeding and 1 only after membrane feeding. • There were exceptions where some medium or heavy gametocytaemias did not lead to infection in mosquitoes whereas some low gametocytaemias led to an infection. • This is the first comparison of the two methods using several different volunteers. • Overall, direct feeding gave better results in terms of infection than membrane feeding. • A weak relationship between gametocyte density and infectiousness of an individual to mosquitoes was observed.

2001	Awono Ambene *et al.*, 2001	Dakar, Senegal	To compare direct and membrane feeding	• Direct and membrane feeding • *P. falciparum* gametocyte carriers were used. • 80 female *An. arabiensis* were used for membrane feeding and 70 mosquitoes for direct feeding.	• 71% of gametocyte carriers were infective for mosquitoes. • The geometric mean number of oocysts per mosquito was higher in membrane feeding. • Infection of mosquitoes was similar with both methods.

As indicated in table 1.8, some of the earlier studies used direct feeding and some used membrane feeding. A comparison reveals both techniques can be used to evaluate human infectiouness to mosquitoes but the studies showed that direct feeding generally gives higher levels of infection than membrane feeding. However, membrane feeding is very useful for experiments in which a researcher needs to add some substance to the blood or to examine the number of gametocytes before feeding mosquitoes on the blood. In the present study, it was decided to use the direct feeding approach for the following reasons:

1. Direct feeding is a closer approximation to the process of natural infection.
2. The limited budget of this project would not have supported membrane feeding equipment or the employment of a nurse to draw blood from the patients.
3. Some hill-tribe people believe that their guardian spirits do not allow the drawing of blood.

1.8 Studies of asymptomatic malaria infections

Where people have malaria parasites in their blood but, due to immunity, the parasite density is so low as to escape slide detection and make diagnosis difficult, such infections may persist because most people believe that if the fever is not severe, it will soon disappear and they therefore take no medication. Thus many parasitaemic adults do not seek blood examination from malaria workers or a malaria clinic although they have malaria fever.

Such asymptomatic or nearly asymptomatic malaria infections could be a significant health problem from two aspects. First, there may be adverse effects to the individual from harbouring such an infection. Asymptomatic infections may be in the process of being controlled and eliminated, but alternatively they may also be capable of conversion to clinical disease. Second, there are the consequences to the general population of the presence of individuals harbouring such infections. The obvious danger is that such asymptomatic carriers are a reservoir of infection to mosquitoes, especially those residing near forested regions, mountains and foothills where migrants and indigenous people reside in close contact, and areas in which control measures, such as DDT house spraying, have been stopped for a long time.

In African endemic areas, only a small proportion of malaria infections reach a sufficiently high parasitaemia to cause an acute illness; the remainder produce a low-level, asymptomatic parasitaemia which may last for weeks or months (Gilles, 1986). Asymptomatic cases seem to play a major role in maintaining parasitaemia, especially in children who are continually exposed to malaria infection, and it is generally believed that repeated exposure is required for acquisition of protective immunity (McGregor *et al.*, 1956).

Several studies in Africa indicated that children less than six months of age, experience few or no episodes of clinical malaria (Garnham, 1949.; MacDonald, 1950; Bruce-Chwatt, 1952; Biggar *et al.*, 1980; Greenwood *et al.*, 1987a; Sehgal *et al.*, 1989; Brabin, 1990; Marsh *et al.*, 1995; Wagner *et al.*, 1998). Parasite prevalence

is lower in the first year of life than in subsequent years and is especially low in children less than 3-4 months old (Sehgal *et al.*, 1989; Hogh *et al.*, 1991; Petersen *et al.*, 1991; Akanmori *et al.*, 1995; Gottschau and Hogh, 1995; Snow *et al.*, 1996). In these very young babies there are several possibly relevant factors such as the presence of foetal haemoglobin (HbF) (Allison, 1954; Pasvol *et al.*, 1976; 1977), low uptake of dietary p-aminobenzoic acid (PABA) in breast milk (Maegraith *et al.*, 1952; Hawking, 1953; Jacobs, 1964) and the transplacental transfer of maternal malaria-specific antibodies (Brabin, 1990; Carlier, 1995). These factors may account for the low incidence of clinical malaria in infants by inhibiting parasite growth, preventing the development of high parasitaemia and thus protecting against clinical disease so that infection in infants is usually asymptomatic or uncomplicated. This pattern is different from that in areas of low and unstable transmission such as Thailand where all age groups are at risk, although severe disease is more common in children and pregnant women (Nosten *et al.*, 1991).

Infected individuals without obvious malaria symptoms may still have abnormal features such as anaemia, splenomegaly or nephrotic syndrome (Greenwood *et al.*, 1987a). Furthermore, Fleming (1981) reported that in semi-immune women, malaria infections during pregnancy rarely cause an acute febrile illness of the type seen in children, but can contribute to anaemia in pregnancy and, occasionally, cause severe life-threatening haemolytic anaemia. McGregor *et al.* (1983) also reported that asymptomatic infections in the mother could cause parasitisation of the placenta. This is important because it leads to lowering of the birth weight that has an adverse effect on child survival.

Roper *et al.* (1996) reported from Sudan that PCR positive samples taken outside the transmission season occurred more frequently among those with experience of a clinical infection during the transmission season. It is possible that these asymptomatic infections were a chronic continuation of the initial infection despite treatment. This phenomenon has been observed in Thailand and is considered to be caused by a persistence of drug-resistant parasites (Snounou *et al.*, 1993a; 1993b).

Elhassan *et al.* (1995) in Sudan found that about 90% of a study population were exposed to *P. falciparum*, but only 33% of the studied individuals experienced clinical malaria. This implies that the population has a higher degree of immunity than expected in an area of low and seasonal endemicity and that many individuals control infections to such an extent that they are normally asymptomatic with low parasitaemias that are difficult to detect.

In Pakistani Punjab, from one-third to one-half of individuals with *P. falciparum* parasitaemia and two-thirds of those with *P. vivax* parasitaemias were oligo-symptomatic or asymptomatic (Strickland *et al.*, 1987). Rajagopalan *et al.* (1990) reported results from a mass blood survey in the Koraput district, Orissa, India indicating that asymptomatic carriers ranged from 61 to 85 per cent and gametocyte carriers ranged from 9 to 25 per cent among all positives. This study also showed that parasitic load in the community is very high in hilltop and foothill villages and most patients are asymptomatic there. The author suggested that the continuation of asymptomatic infection is due to inadequate surveillance and treatment or due to a certain degree of immunity in the population.

In Thailand, prevalence of asymptomatic malaria of both species has been reported. Kamol-Ratanakul *et al.* (1992) showed that in Chonburi Province, southeast of Bangkok, 77% of malarial infections were asymptomatic (12.6% of the surveyed population), and most of the studied subjects were semi-immune and usually lived in an endemic area. The same author (Kamol-Ratanakul *et al.*, 1994) gave results from a study in a rural area of Eastern Thailand, along the Thai-Cambodian border. Among a cross-sectional survey, 63 out of 451 were positive and 42 of those were asymptomatic (about 9.9% of the surveyed population). From these studies it can be concluded that the prevalence of asymptomatic infections was very low and not so common in Thailand as in highly endemic parts of Africa. The studies on asymptomatic malaria mentioned above were carried out in Eastern Thailand. The work described in the present thesis involved mass blood surveys for asymptomatic malaria on the north western border of Thailand.

1.9 Research questions and objectives of the study

The main objective of the present study was to investigate in northern Thailand how important asymptomatic or oligo-symptomatic malaria may be, especially as a reservoir of infection to mosquitoes. There are many problems in interpretation of earlier studies in Thailand of asymptomatic malaria such as the fact that blood examination may have been done during the incubation period, in the last period of fever, in the period before starting a fever and some criteria used in the these studies were ill-defined or different. Moreover, some studies were done in the transmission season and others not. In the present study the surveys were at two different times of the year. Direct feeding of mosquitoes on consenting individuals was used to investigate the infectivity of parasites from asymptomatic malaria in village populations and symptomatic malaria cases at a malaria clinic in Muang district in Mae Hong Son province in the Northwest of Thailand. The study was designed to determine whether the reservoir of infection of vectors was mainly in people ill enough to go to the clinic or whether it was mainly in those with slight or no malaria symptoms who remained in the villages.

1.9.1 Research Questions

1. What is the epidemiology of asymptomatic *P. falciparum* and *P. vivax* malaria in healthy people in the study area?
2. What is the distribution of asexual and gametocyte parasite density in asymptomatic malaria?
3. Can the parasites from asymptomatic persons develop in *Anopheles* mosquitoes as well as those from symptomatic persons?
4. What is the percent positive and density of oocysts in mosquitoes fed on the blood of asymptomatic and symptomatic individuals?
5. How important is asymptomatic malaria in maintaining transmission?

1.10 Study area and population

1.10.1 Northern Thailand

The 17 provinces of northern Thailand are mountainous and have a population of over 12 million (Ministry of Interior, 1998). These provinces border Myanmar to the North and Northwest, and Laos in the Northeast (Figure 1.17).

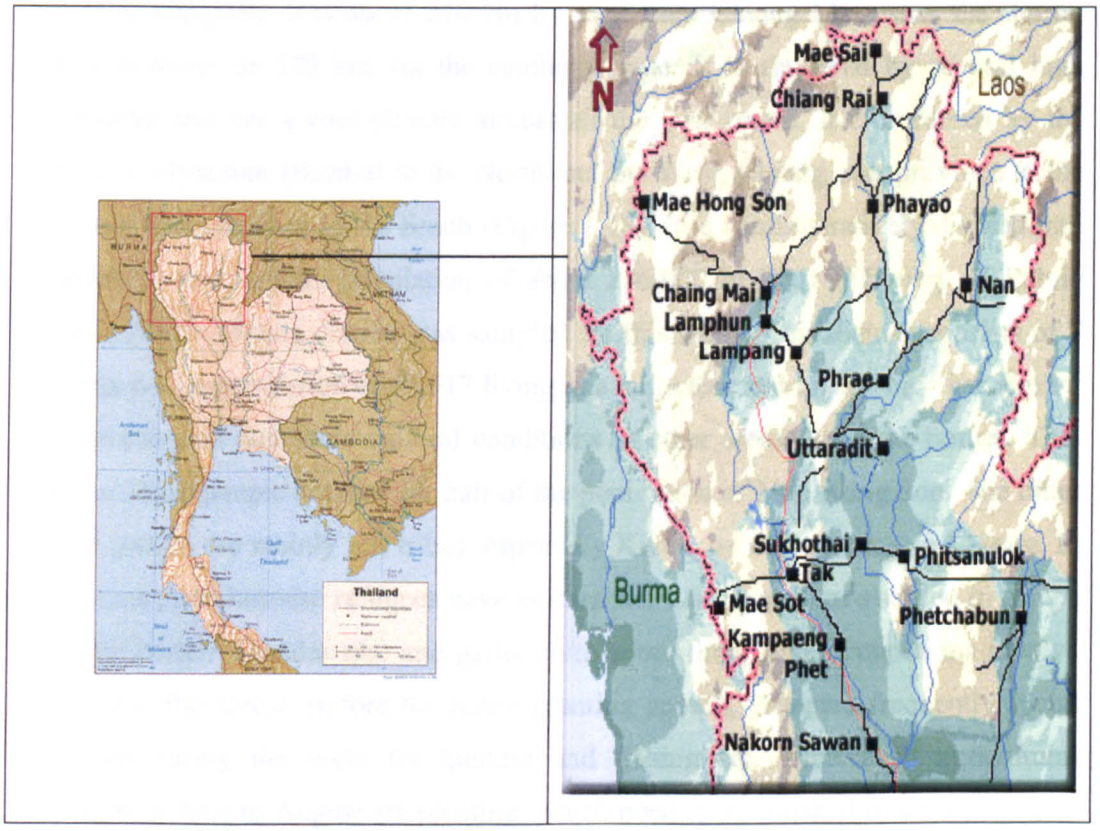


Figure 1.17 Map of Northern Thailand

1.10.2 Mae Hong Son Province

Mae Hong Son is a province situated in Northwestern Thailand. It is sometimes called " City of three mists", because it is set deep in a mountain valley. In November and December it has morning mists, in February and March it has the smoke from burning down the rice stubble and in May to July it has heavy rainfall. Mae Hong Son was also called "Siberia of the North" because, until quite recently,

there were no paved roads and the only connection to the outside was via an elephant path. However, since the paved road and the airport came to this town its economy is booming and tourism has become important because of its beautiful location surrounded by mountains.

This province has an area of 2,681 square kilometres and is located about 925 km from Bangkok. It is about 270 km by road from Chiang Mai using the shorter northern route, or 370 km via the southern route. It is sheltered by several high mountains and has a cool climate almost all the year round. It is bordered by the Union of Myanmar (Burma) to the North and the West, Chiang Mai province to the East and Tak province to the South (Figure 1.18). It is administratively divided into 7 districts, with a total population of about 236,000 in 2001 (Ministry of Public Health, 2001). Muang district was sampled for this study. This district consists of 7 cantons with a population of 33,517 living in a large forested area, which has similar epidemiological and socio-cultural conditions as other areas along the border. Thai Yai (or Shan) people account for half of the population of Mae Hong Son. The other ethnic groups are mainly hill tribes, especially Karen, as well as Lisu and Mong. In the recent past Burmese refugees have crossed the border and have settled in camps near the border. Besides rice and garlic cultivation, their main occupations involve going into the forest. Before the major planting season, villagers frequently spend time out during the night for hunting and fishing etc. Almost all agricultural activities in June to August are planting, which need to be finished in a short time to allow the crop to get enough rain for good yields. All family members who can work are needed for this task. After this time, some of them have more time to do other activities including forest activities or work on the farm not far from their residence and they do not need to stay at their farm hut. They go to the farm very early in the morning and walk back home late in the evening. In the process they are exposed to *An. minimus s.l.*

Factors in this area which might be expected to affect malaria include the level of human and economic activities along forested, mountainous international boundaries, and a recent reduction in vector control coverage due to the Asian

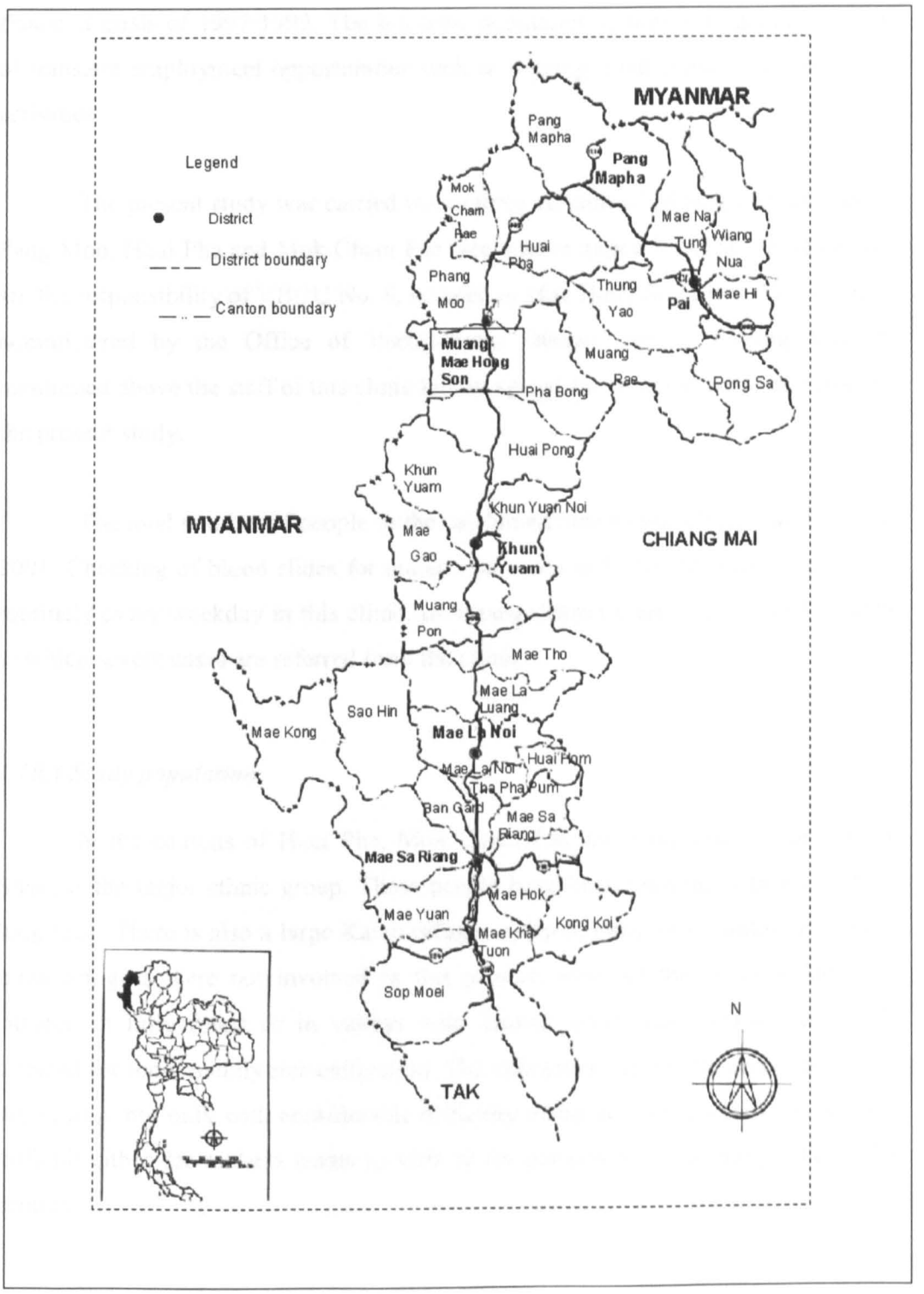


Figure 1.18 Map of Mae Hong Son Province, Thailand

financial crisis of 1997-1999. The hill-tribe population is highly migratory because of transient employment opportunities such as logging, road construction and other activities.

The present study was carried out in three cantons of Muang district, namely Pang Moo, Huai Pha and Mok Cham Pae (see map in annex 3.2-3.5). These cantons are the responsibility of VBDU No. 8, situated in Mae Hong Son town. This Clinic is administered by the Office of Vector-borne Disease No. 2, Chiang Mai. As mentioned above the staff of this clinic kindly agreed that it could serve as a base for the present study.

The total number of people in the catchment area of this clinic was 21,673 in 2001. Checking of blood slides for malaria parasites and free treatment is provided routinely every weekday in this clinic. In Muang district there is a 150 bed hospital to which severe cases are referred from the clinic.

1.10.3 Study population

In the cantons of Huai Pha, Mok Cham Pae and Pang Moo, Thai Yai (or Shan) is the major ethnic group. These people have been resident in this area for a long time. There is also a large Karen refugee camp in Pang Moo canton. However, these refugees were not involved in this project. Most of the study villages are situated in hilly forest or in valleys with limited level areas which are usually terraced for food, mainly rice cultivation. The villages are generally accessible in the dry season, but only with considerable difficulty when the rain starts. This makes it difficult either for malaria teams to visit or for patients to go to the public health centres.

CHAPTER 2

MATERIALS AND METHODS

2.1. Mosquitoes

2.1.1 Mosquito maintenance

A colony of *An. minimus* species A has been kept in the insectary of the Office of Vector-borne and Disease Control No. 2, Chiang Mai, for many years. Before starting the project, the number of mosquitoes was increased by using artificial mating. The colony was maintained at 28°C ± 2°C and 70-90% relative humidity (RH). Both photoperiod and light intensity affect development in mosquitoes. Thus the photoperiod was controlled in the insectarium with lights on at 08.00 hr and off at 17.00 hr. A standard fluorescent light provided an approximation to "daylight". At night time all lights were turned off with no attempt to simulate "dusk" and "dawn" as in some insectaria. There was a constant supply of 5% sucrose for adult mosquitoes held in wire-frame net cages (size 30 x 30x 30 cm). After the age of five to seven days, female mosquitoes were given two blood meals a week using guinea pigs.

Two to three days after blood meals, small bowls of water, which were lined with filter paper in order to prevent drying and desiccation of eggs, were placed in the cages to allow oviposition. Eggs were collected and placed for hatching in plastic trays containing approximately 1 litre of local well water. Plastic straws were floated in a triangular shape in order to confine the eggs and prevent them spreading and adhering to the edges of the tray. After hatching, the larvae were transferred into plastic trays containing 2 litres of tap water.

In this insectarium, we attempted to standardise the size of larvae since several studies showed the impact of mosquito size on the intensity of infection. The larvae were fed twice a day on small quantities of dog food with a check made that all the previous food had been consumed before more was supplied. Larval density was limited to 400 per 23 x 30 cm tray. Larvae were fed daily with dog food (liver or beef flavour purchased from a pet shop). The pupae were harvested daily and transferred into small plastic bowls which were placed in the colony cages for

emergence. When adult mosquitoes were required for infection experiments, bowls of pupae were transferred into separate cages.

2.1.2 Transport of adult mosquitoes to the field

Adult females aged 4-6 days were used for feeding on volunteers with parasitaemia. 20-30 mosquitoes were put into paper cups covered with mosquito netting. They were fed with cotton moistened with 5% sucrose and then were transferred to the field in a polystyrene box and carried on the regular flight to Mae Hong Son Province from Chiang Mai Province. The flight time was 25 minutes. During transportation, it was ensured that the mosquitoes were not left in direct sunlight. They were kept at Mae Hong Son Province for up to 5-7 days while volunteers with parasitaemia were sought. During the waiting period, the cotton with 5% sucrose was changed every two days.

2.2 Subjects

2.2.1 Recruitment of subjects from mass blood surveys in the villages (Figure 2.1).

Mass blood surveys were conducted twice a year for all age groups during the cool dry (November-March), and rainy seasons (May-August). The surveys were carried out in 30 villages in the three cantons. Before the blood collections and interviews, village chiefs were contacted at least two days in advance in order to encourage local co-operation and malaria staff also explained the purpose of the blood collections. Then village chiefs announced the time, date and place of meeting using the village loud speaker towers. Normally these activities took place at the centre of the village, i.e. the temple or school. The target groups were interviewed prior to taking blood smears. Only data from these interviewed people have been used for further analysis.

The baseline data for this part of the study (name, address, age, sex, occupation, mobility, impregnated bed net use and other mosquito protection methods etc.) were collected by using a structured questionnaire (Annex 2.1). The

amount of rainfall per month, average monthly temperature and relative humidity were collected from routine records.

Asymptomatic subjects were eligible for recruitment if they fulfilled all the following criteria:

- Slide positive
- Had a core body temperature ≤ 37.4°C
- No reported fever on the day of taking blood
- No other malarial symptoms in previous weeks.
- Had not taken any anti-malarial drug (self-reporting)

After slides were examined for parasites, the people who were slide positive and aged more than 15 years were recruited. The Thai ethical committee did not allow direct feeding of mosquitoes on children aged less than 15 years. The patient was informed about the purpose of the study and, if they agreed, a consent form was signed and patients were interviewed. Then the core body temperature was measured with a Gentle temp Omron MC-505, thermometer (Omron Healthcare Singapore Pte, Ltd) which instantly reads radiant heat from the eardrum. As a check on reliability, the temperature was read from the left ear then the right ear and then back to the left ear. The three readings generally agreed within ± 0.1°C and the mean of the temperatures was calculated. Then thick blood smear was taken by finger prick and samples were taken on to glass fibre filter paper for later PCR studies and, after that, a direct feeding was carried out as described in section 2.3.1. Thick blood smears were air dried and stained with 4% Giemsa and one hundred microscopic oil immersion fields were systematically examined in the field for malaria parasites by trained microscopists. On return to Chiang Mai, all positive thick blood films were counted for parasitaemia per 200 WBC. All parasite densities were assessed relative to numbers of white blood cells. Five randomly chosen sectors of each slide were assessed for sexual and asexual stages until 200 WBC had been counted. Thus a total of 1,000 WBC was observed. Using the standard conversion factor of 8,000 WBC per microlitre, this indicated that 0.125 µl of blood was observed. Asexual and

gametocyte stages were counted separately. Parasite density was expressed as parasites per μl, assuming 8 x 10^3 WBC/μl (Wilcox, 1960).

2.2.2 Recruitment of patients at the clinic (Figure 2.1)

Study patients were enrolled from the Vector-borne Disease Control Unit No. 8, Muang District, Mae Hong Son Province. In this Clinic blood films are taken for checking for malaria. Patients who had presented with clinical malaria and/or an asexual parasitaemia were informed about the purpose of the study and asked to participate in the study. If they agreed, then the procedures were followed as described in section 2.2.1

Asymptomatic patients were eligible for recruitment if they fulfilled all the criteria for asymptomatic infection as described for the subjects from the village surveys.

Symptomatic patients were recruited if they fulfilled the following criteria:

- Slide positive
- Had a core body temperature > 37.4°C and/or
- Reported fever on the day of taking blood

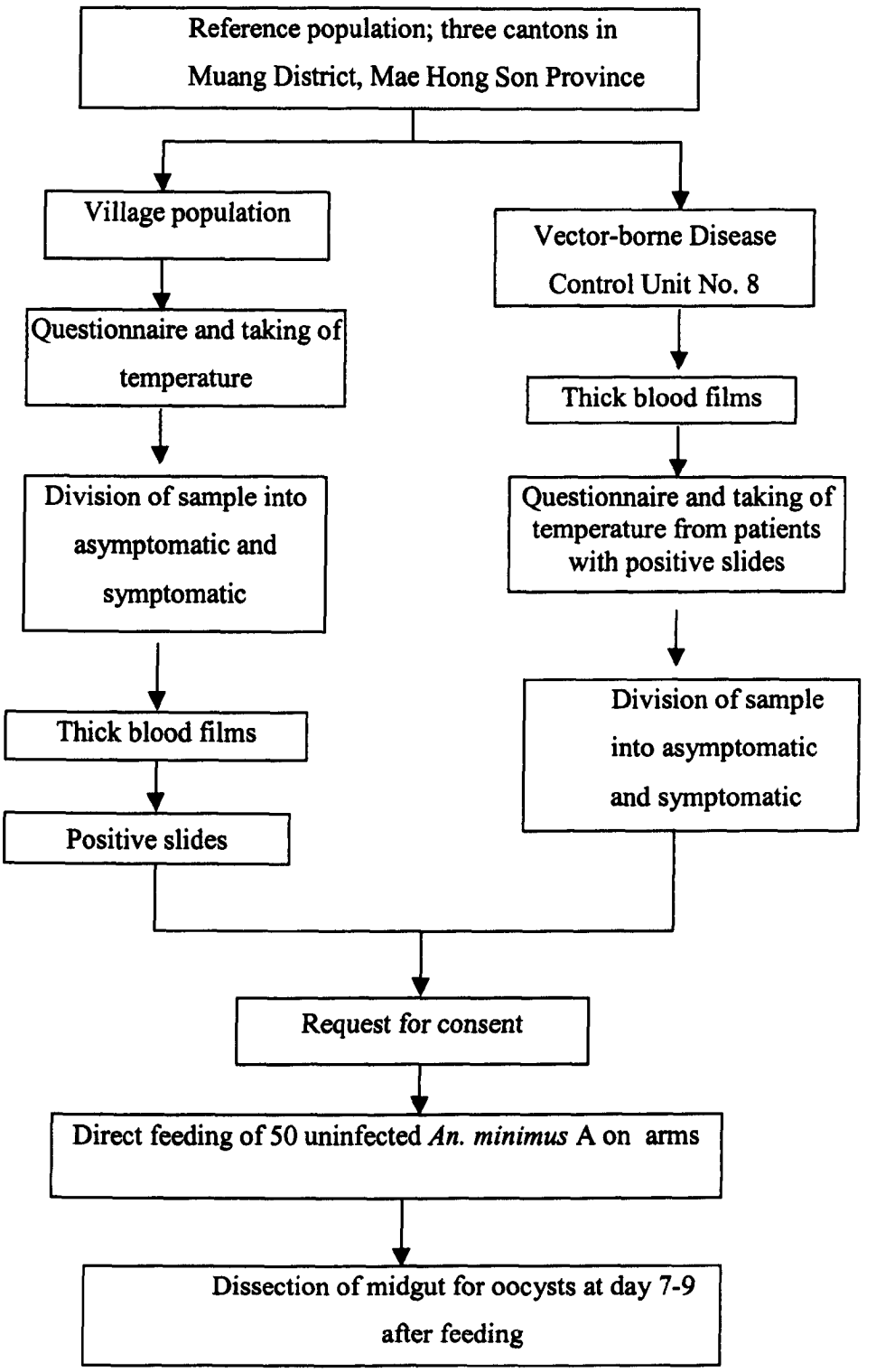


Figure 2.1 Flow chart of data collection

2.3 Mosquito feeding and dissection

2.3.1 Direct feeding

Before feeding, patients were asked about mosquito allergy and their arms were cleaned with 70% alcohol and then 50 laboratory-bred female *An. minimus* A (age 4-6 days) which had been starved for 9-12 hr were allowed to feed on patients' arms for 30 minutes (Figure 2.2). After feeding was completed, the patients' arms were cleaned with alcohol, rubbed with an anti-histamine cream and anti-malarial drugs were given to all positive cases by a malaria worker.

Two hours after feeding the unengorged mosquitoes were removed from the cups using a sucking tube and destroyed, leaving only fully engorged mosquitoes in the cup. For security, the cups with engorged mosquitoes were covered with an extra layer of mosquito net and then were supplied with cotton soaked in 5% sucrose. The mosquitoes were brought back to the Chiang Mai insectarium by the regular plane service and maintained at 25° to 27°C and 70-80% relative humidity with permanent access to sucrose solution and without any further blood meals.

Figure 2.2 Mosquito feeding

2.3.1 Dissection and oocyst counts

Mosquitoes at 7-9 days post-feed were anaesthetized with ether and the wings and legs were removed. Midguts were dissected out on glass slides in a drop of 0.85% NaCl and examined at a magnification of 40x. The number of oocysts present on the midgut of each mosquito was counted (Eyles, 1950). To avoid contamination in future PCR studies, a new needle was used for each mosquito. After examination for oocysts, each midgut was transferred to an Eppendorf tube which contained 0.85% NaCl. Positive and negative midguts were kept separately in each Eppendorf and kept at -20°C to use for PCR work.

The mosquitoes were not kept alive until they could have developed sporozoites because, especially from an area with multi-drug resistant *P. falciparum*, such mosquitoes were dangerous to keep in the laboratory.

2.4 Questionnaire

The questionnaire was adapted from one used by Aramrattana (1993). A pilot trial to validate the questionnaire was performed before using it in the project. As a result some leading or confusing questions were revised. Trained interviewers who can speak the languages of the study population conducted the interviews. Approximately 60% of those people can speak Thai but the remainder speak Karen, Paow, Mong and Laho. To check the accuracy of reported last experiences of malaria, we re-checked at the health facilities where the interviewees stated that they went for malaria treatment.

2.5 Research team

Two blood collectors, two microscopists and two interviewers were involved in each survey. These staff was from the Vector-borne Disease Control Unit No. 8, Mae Hong Son and one driver was provided by the Vector-borne Disease Control Centre No.21. The candidate was involved in all surveys which were carried out in 30 villages. She conducted the interviews in those villages where the population can speak Thai. When the microscopists reported positive slides, the candidate re-checked the slides before recording the parasite species. The candidate was also responsible for and involved in all stages of the entomological work.

2.6 Quality Control of data

Throughout the period of the study, 20 % of the negative blood smears and all positive slides which had been examined by the microscopist of the Malaria Clinic in Mae Hong Son Province were chosen randomly and re-examined by a team at the Office of Vector-borne Disease Control No.2, Chiang Mai. All cases of positive slides without visible gametocytes and which led to oocyst production were re-checked again by an expert team at the London School of Hygiene and Tropical Medicine to check whether a few gametocytes might have been present but were initially missed.

2.7 Ethical Considerations

Direct feeding has been used in previous studies (e.g. Graves *et al.*, 1988; Sattabongkot *et al.*, 1991) and people preferred direct feeding rather than taking of venous blood with a syringe for membrane feeding. As already stated, patients were informed of the purpose of the study and, if they agreed, a consent form was signed before feeding. The safety of direct feeding of mosquitoes on the arms of asymptomatic individuals was ensured by using laboratory-bred uninfected *An. minimus* A. Patients were informed that in case of allergy or hypersensitivity, they

could voluntarily withdraw from the feeding at any time. However, during this study nobody refused to participate or demanded that feeding was stopped.

This study was approved by the Thai Ethics Committee and the Ethics Committee of London School of Hygiene and Tropical Medicine.

2.8 Data management and statistical analysis

Structured questionnaires were used in the village surveys. After checking for missing information, the questionnaires were coded and double-entered into a computerised form, validated and analysed using Epi-info version 6.04. This program and Stata version 6 was used for analysis. The significance of differences in categorical data was examined using chi-square tests unless any expectation on the null hypothesis was <5, in which case Fisher's exact test was used. Where data occurred in strata which may have been heterogeneous the Mantel Haenszel χ^2 was used. The database of mosquito feeding included: 1) the densities of gametocytes and trophozoites, 2) number of infected mosquitoes, and 3) mean number of oocysts per infected mosquito. These parameters have been used by several authors (Muirhead-Thomson, 1957; Graves *et al.*, 1988; Sattabongkot *et al.*, 1991; Boudin *et al.*, 1993). The distributions of such data are generally strongly positively skewed. To approximately normalise the data logarithmic transformation was used. When any data points were zero Williams' mean was used, i.e. [antilog [$\sum$log (x+1)/n]]-1 for the purpose of reporting a measured of central tendency. However, statistical inference (t-test) was conducted on the log-transformed (normalised) data.

CHAPTER 3

OBSERVATIONS ON THE EPIDEMIOLOGY OF MALARIA IN VILLAGE SURVEYS AND THE MALARIA CLINIC

3.1 Village surveys

3.1.1 Results from the collection of blood smears

3.1.1.1 Age and sex distribution

Mass blood surveys (without regard to the occurrence of malaria symptoms) were carried out primarily to find malaria infected individuals for the mosquito infection studies (Chapter 4). However, the data collected were of interest in providing information on endemicity in village populations in the study area. The surveys were carried out from November 1999 to March 2000 and again from May to August 2000 in 30 villages of three cantons: Pang Moo, Huai Pha and Mok Cham Pae. The populations of the villages in the study ranged from 44 to 2,328 with an average village population of 420. The number of blood smears taken per village was from 33 to 559. A total of 7812 blood smears were taken and examined. During the two surveys blood smears were taken from 46.8% (4320 out of 9227) of the total population during the first survey and 42.2% (3492 out of 8274) during the second survey.

From the population census in 1999, adults (age ≥15 years) represented 73.5% of the population of the three cantons (Table 3.1).

Table 3.1 Age distribution of the whole population in the study area based on population census in 1999.

Cantons	No. of households	Population	Age (Years)			
			< 1	1-4	5-14	>15
Pang Moo	1,988	8,628	113	438	1,873	6,204
Huai Pha	710	2,442	36	153	463	1,790
Mok Cham Pae	742	3,174	29	235	610	2,300
Total	6,375	21,734	204 (0.94%)	1,051 (4.84%)	4,506 (20.73%)	15,973 (73.49%)

More females than males (52.5% vs. 47.4%) were included in this study. The ratio, however, was not consistent for all age groups (Table 3.2 & 3.3). The proportion of males was lower in the 15-29 and the 30-29 age groups in the first survey because it was during a harvest season so most men were working in the fields outside the villages. They left home early in the morning and came back late at night so their time at home and our visits did not match. However, the proportion of males of those age groups was higher during the second survey. The proportion of adults (age ≥15 years) was higher than that of children in both surveys (62.2% in the first survey and 64.9% in the second survey). This is similar to the census population shown in Table 3.1.

Table 3.2 Age and sex distribution of the study population in the first survey.

Age group	**No. & % of males in each age group**	**No. & % of females in each age group**	**No. & % of whole population in each age group**	**Male:female ratio**
0-4	175 (8.6%)	190 (8.3%)	365 (8.4%)	0.92:1
5-9	295 (14.5%)	336 (14.7%)	631 (14.6%)	0.88:1
10-14	319 (15.7%)	318 (13.9%)	637 (14.8%)	1.00:1
15-29	427 (21.1%)	524 (22.9%)	951 (22.0%)	0.81:1
30-59	633 (31.2%)	740 (32.3%)	1373 (31.8%)	0.86:1
≥60	181 (8.9%)	182 (7.9%)	363 (8.4%)	0.99:1
All age groups	2030 (100%)	2290 (100%)	4320 (100%)	0.88:1

Table 3.3 Age and sex distribution of the study population in the second survey.

Age group	No. & % of males in each age group	No. & % of females in each age group	No. & % of whole population in each age group	Male:female ratio
0-4	152 (9.1%)	141 (7.8%)	293 (8.4%)	1.08:1
5-9	197 (11.7%)	250 (13.8%)	447 (12.8%)	0.79:1
10-14	239 (14.3%)	247 (13.6%)	486 (13.9%)	0.97:1
15-29	339 (20.2%)	382 (21.1%)	721 (20.6%)	0.89:1
30-59	608 (36.3%)	658 (36.2%)	1266 (36.3%)	0.92:1
≥60	142 (8.4%)	137 (7.5%)	279 (7.9%)	1.04:1
All age groups	1677 (100%)	1815 (100%)	3492 (100%)	0.92:1

3.1.1.2 Parasite prevalence

The positive cases of malaria infection in each survey are shown in Table 3.4. The parasite rate, defined as the proportion of the sample with blood films positive for any species of malaria parasite, was 0.6% (28/4,320) for the first survey and 0.8% (29/3,492) for the second survey. However, the parasite prevalence was not significantly different between the two surveys (χ^2 with Yates' correction = 0.65, P = 0.42). A slight excess of *P. vivax* was seen in the two surveys at different seasons.

Blood smear positivity has been further divided into high and low parasitaemia by classifying those films with parasite densities >3,690/μl [equivalent to >99 parasites/200 WBC assuming a mean of 8,000 WBC/μl (Wilcox, 1960)] as "high" parasitaemia and those with 40-3,960/μl (equivalent to 1-99 parasites/200 WBC) as "low" parasitaemia. Using this cut-off point approximately divides the sample of positive blood slides into two halves. Curtis *et al.* (1998) found that in Tanzanian children parasitaemias above this cut-off point were strongly associated with occurrence of fever.

Table 3.4 Number and positive cases from the two surveys.

Surveys	Results				Total
	Negative slides (%)	*P. falciparum* (%)	*P.vivax* (%)	Mixed infection (%)	
1st survey (Nov' 99-Mar'00)	4292 (99.3)	12 (0.3)	15 (0.4)	1 (0.0)	4320
2nd survey (May-Aug' 00)	3463 (99.2)	12 (0.3)	17 (0.5)	0 (0)	3492
Total	7755 (99.3)	24 (0.3)	32 (0.4)	1 (0.0)	7812

3.1.1.2.1 Parasite rate in each canton

The number of blood slides taken in each canton and prevalence of the two parasite species in the three cantons are shown in Table 3.5 and Figure 3.1, respectively. The prevalence of all species was 0.9% in Pang Moo, 0.8% in Huai Pha and 0.5% in Mok Cham Pae canton. However, the difference between the prevalence of cases in the three cantons was not statistically significant ($\chi^2 = 2.06$, $df = 2$, $P = 0.35$). The proportion of *P. falciparum* and *P. vivax* was similar in the three cantons. Individual villages within the cantons appeared to differ markedly in prevalence with many having zero cases and one having 9 cases (annex 1.1).

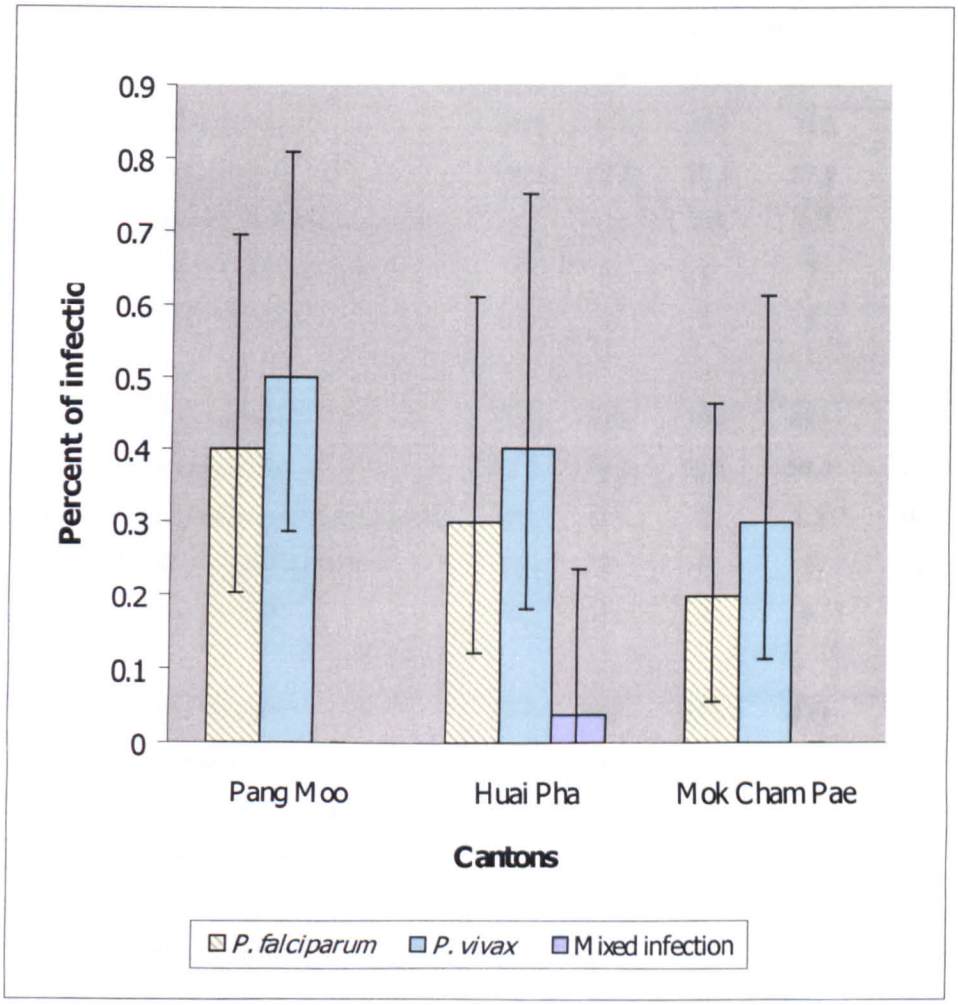


Figure 3.1 Parasite prevalence in three cantons, by species with exact 95% confidence limits based on the binomial distribution.

Table 3.5 Blood smears taken and positive cases in three cantons and the totals in each age group.

Cantons		Age group (years)					
		0-4	**5-9**	**10-14**	**15-29**	**30-59**	**60+**
Pang Moo	No. smears	239	476	491	712	913	198
	Female (%)	48.5	53.8	51.5	57.9	52.3	52.5
	Blood smear positive (%)	0.4	1.1	0.4	1.7	0.7	0
	No. *P. falciparum*	0	1	1	7	3	0
	No. *P. vivax*	1	4	1	5	3	0
Mok Cham Pae	No. smears	230	336	377	481	812	208
	Female (%)	54.5	55.1	51.5	54.5	57.6	46.6
	Blood smear positive (%)	0.4	0.3	0	1.3	0.4	0.9
	No. *P. falciparum*	0	0	0	3	1	1
	No. *P. viax*	1	1	0	3	2	1
Huai Pha	No. smears	189	267	255	479	913	236
	Female (%)	47.1	54.7	46.3	48.4	49.6	50.0
	Blood smear positive (%)	1.1	1.5	1.6	0.6	0.4	0.4
	No. *P. falciparum*	0	2	1	2	2	0
	No. *P. vivax*	2	1	3	1	2	1
	Mixed infection	0	1	0	0	0	0
Total	No. smears	658	1079	1123	1672	2638	642
	Female (%)	50.2	54.4	50.3	54.2	53.0	49.7
	Blood smear positive (%)	0.6	0.8	0.6	1.3	0.5	0.5
	No. *P. falciparum*	0	3	3	12	6	1
	No. *P. vivax*	4	5	4	9	8	2
	Mixed infection	0	1	0	0	0	0

3.1.1.2.2 Age related differences in parasite rate

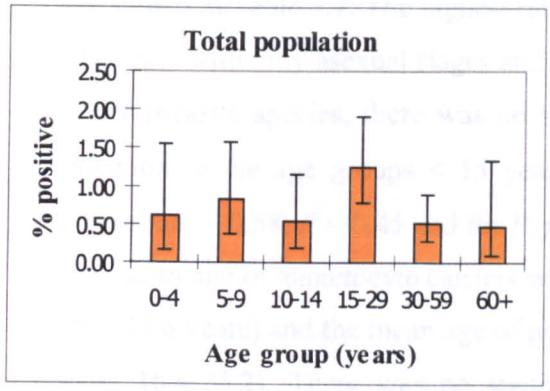
The age-specific distribution parasite prevalence is shown in Figure 3.2. The prevalence in children under five years of age was 0.6% (4/658). The age group 15-29 years had the highest observed prevalence in two cantons but, in Huai Pha, prevalence was higher in the 5-6 and 10-14 year age group than in adults. There was a borderline significant difference between the prevalence of cases in each age group for the three cantons combined ($\chi^2 = 9.34$, $df = 5$, $P = 0.09$) with an overall peak in the 15-29 year group. The malaria prevalence was higher in males than females. This difference was significant from a Mantel-Haenszel χ^2 test stratified by age group ($\chi^2_{MH} = 8.15$, $P = 0.004$).

Table 3.6 Age-specific malaria prevalence

Sex	Age group						Total
	0-4	5-9	10-14	15-29	30-59	60+	
Male	2/327 (0.6%)	4/492 (0.8%)	3/558 (0.5%)	18/766 (2.3%)	9/1241 (0.7%)	2/323 (0.6%)	38/3707 (1.0%)
Female	2/331 (0.5%)	5/586 (0.9%)	3/565 (0.5%)	3/906 (0.3%)	5/1398(0.4%)	1/319 (0.3%)	19/4105 (0.5%)

n = number blood smears in each age group

A



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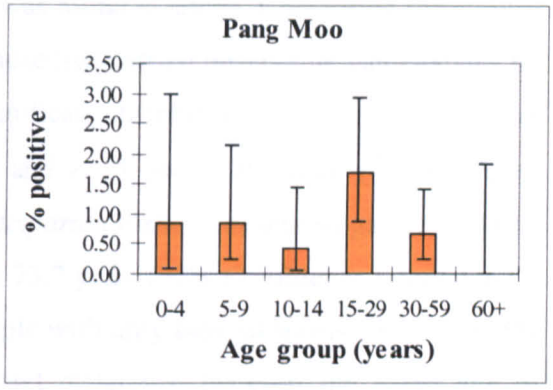

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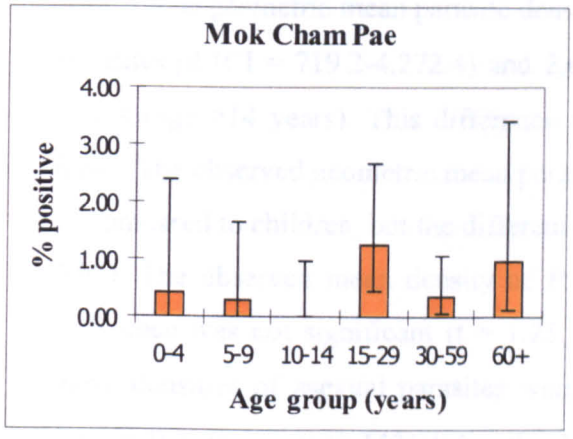

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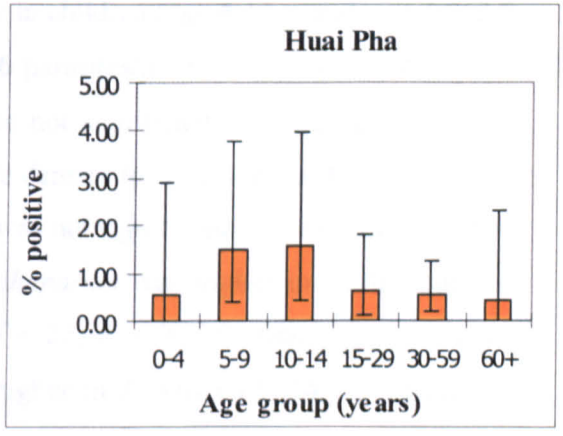

Figure 3.2 Percentage parasite positive (with 95% confidence limits) in total population (A) and three cantons; Pang Moo (B), Mok Cham Pae (C) and Huai Pha (D). Notice the change of scale in the graphs.

3.1.1.3 Parasite and gametocyte densities

Age specific positivity rates for *P. falciparum* and *P. vivax* in both surveys are shown in Table 3.7. The highest rate was found in adults. Comparing the number of people with only asexual stages and those that carried observable gametocytes for either parasite species, there was no significant difference between these types of infection in the age groups < 15 years and ≥ 15 years (*P. vivax* χ^2 with Yates' correction = 0.58, $P = 0.45$ and for *P. falciparum* Fisher's exact test gave $P = 0.61$). The mean age of gametocyte carriers was 23.7 years (95% Confidence interval, CI = 15.9-31.6 years) and the mean age of people with only asexual forms was 20.8 (95% CI = 16.4-25.2). There was no significant difference between the mean age of gametocyte carriers and people with asexual parasites only (t = 0.72, $df = 55$, $P = 0.46$).

The geometric mean parasite density in children (age 4-14 years) was 1,752.8 parasites/μl (CI = 719.2-4,272.4) and 2,696 parasites/μl (CI = 1,492.8-4,868.8) for adults (age >14 years). This difference was not significant (t = 0.2, $df = 57$, $P = 0.84$). The observed geometric mean parasite density for *P. vivax* was lower in adults as compared to children, but the difference was not significant (t = 0.85, $df = 30$, $P = 0.96$). The observed mean density of *P. falciparum* was higher in adults, but the difference was not significant (t = 1.25, $df = 22$, $P = 0.23$). Observed geometric mean densities of asexual parasites were higher in *P. vivax* (2,644/μl) infections, than in *P. falciparum* (2,153/μl) but the difference was not significant (t = 0.44, $df = 54$, $P = 0.67$).

Table 3.7 Gametocytes and asexual infection rates of both species by age group

Species	Age group						Total
	0-4	5-9	10-14	15-29	30-59	60+	
Total blood slides	658	1,079	1,123	1,672	2,638	642	7,812
No. positive	4	10	6	21	14	3	57
Proportion positive (%)	0.6	0.9	0.5	1.3	0.5	0.5	0.7
P. falciparum	0	3	2	12	6	1	24 (42.1%)[a]
No. with visible gametocytes	0	2	0	1	4	0	7 (29.2%)[a]
No. with asexuals only	0	1	2	11	2	1	17 (70.8%)[a]
Geometric mean density (parasites/µl of blood)	0	856	948	2,739	2,215	8,400	2,152
P. vivax	4	6	4	9	7	2	32 (56.%)[a]
No. with visible gametocytes	2	2	1	6	2	2	15 (46.9%)[a]
No. with asexuals only	2	4	3	3	5	0	17 (53.2%)[a]
Geometric mean density (parasites/µl of blood)	612	3,428	7,384	2,517	3,452	1,443	2,644
Mixed infection*	0	1	0	0	0	0	1 (1.8%)[a]
No. with visible gametocytes	0	1	0	0	0	0	1 (100%)[a]
No. with asexuals only	0	0	0	0	0	0	0
Geometric mean density (parasites/µl of blood)	0	120	0	0	0	0	120
Overall geometric mean density (parasites/µl of blood)	612	1,700	3,724	2,604	2,813	2,596	2,316

[a] % of positive

* Only one case

Gametocytes of *P. falciparum* were detected in only 7 samples out of 24 slides positive for this species: 2 from children (geometric mean = 236/μl) and 5 from adults (geometric mean = 140/μl). *P. vivax* gametocytes were detected in 5 children (geometric mean = 108/μl) and 10 adults (geometric mean = 368/μl) out of 32 slides positive. The geometric mean gametocytaemias were 163/μl (95% CI = 60-444/μl) and 245/μl (95% CI = 120-500/μl) for *P. falciparum* and *P. vivax*, respectively, but the difference was not significant ($t = 0.71$, $df = 20$, $P = 0.48$). In the one case of mixed infection the gametocytaemia was 40/μl.

The distribution of gametocyte densities of *P. falciparum* and *P. vivax* are shown in Figure 3.3. More than 85% of *P. falciparum* and 80% of *P. vivax* gametocytaemias were at densities ≤ 960/μl.

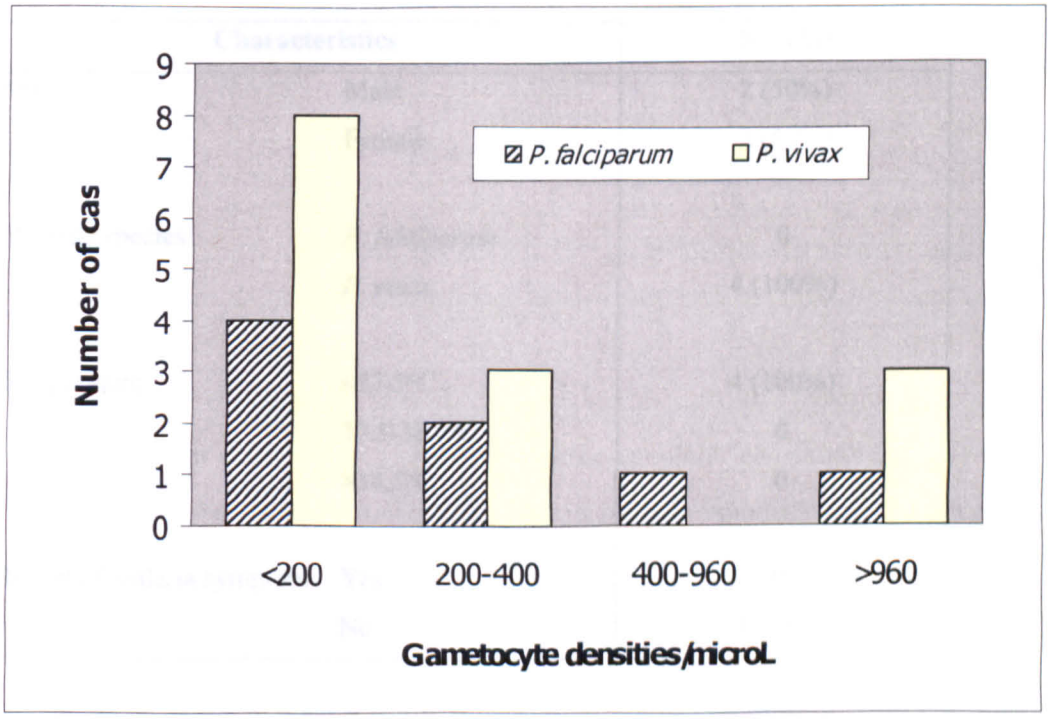

Figure 3.3 Frequency distribution of gametocyte densities in 7 *P. falciparum* and 15 *P. vivax* gametocyte carriers.

3.1.2 Results from interviews with villagers whose blood was sampled

3.1.2.1 Demography of surveyed population

Of the surveyed population of 7812, 5231 (67.0% of donors) were interviewed using a structured questionnaire (annex 2.1).

In the case of the four slide positive individuals among the children aged 0-4 years interviews were conducted, with the help of parents/guardians. None of the other 0-4 year olds were interviewed. The characteristics of those 4 cases are shown in Table 3.8. All of them were infected with *P. vivax* and had body temperature <37.5°C and three of them were reported asymptomatic. These four young children are excluded from further analysis which was on 5227 subjects aged ≥ 5 years.

Table 3.8 Characteristics of infected children aged <5 years.

Characteristics		No. (%)
Sex	Male	2 (50%)
	Female	2 (50%)
Parasite species	*P. falciparum*	0
	P. vivax	4 (100%)
Temperature	<37.5°C	4 (100%)
	37.5-38.5°C	0
	>38.5°C	0
Report of malaria symptom	Yes	1 (25%)
	No	3 (75%)

As shown in Table 3.9 there were slightly more females than males in all age groups. More than 95% of the surveyed population in each age group had a core body temperature below 37.5°C as measured with a Gentle temp Omron MC-505 thermometer placed in the ear. Mild fever (37.5-38.5°C) was twice as common in children as adults (significant difference, χ^2 with Yates' correction = 14.35, $P<0.001$). High fever (>38.5°C) had a prevalence of 1.1% in children but was rare in adults (significantly different, Fisher's exact test, $P = 0.002$). About 10.7% of the surveyed population reported having fever in the previous week. The proportion of adults who had stayed outside the village in the previous month was higher than in children. This can be explained by the fact that most males and some of the females work in rice fields or forests and they sometimes have to stay there for one night or more, especially during in the harvest season. Some work places are very far from their villages so it is impossible to return home every night. Staying overnight in forests exposes adults to more biting by *An. dirus s.l.* than they would experience at home (Somboon *et al.*, 1998).

Table 3.9 Characteristics of the surveyed population of 5227 classified by age group.

	Age group (years)		
	5-14	15-59	60+
Total no. smears:	623	4000	604
Female	319 (51.2%)*	2135 (53.6%)	304 (50.3%)
Core body temperature:			
< 37.5°C	592 (95.0%)	3928 (98.2%)	592 (98.0%)
37.5°C–38.5°C	24 (3.9%)	64 (1.6%)	9 (1.5%)
>38.5°C	7 (1.1%)	8 (0.2%)	3 (0.5%)
Reported fever in the previous week:	63 (10.1%)	428 (10.7%)	70 (11.6%)
Stayed outside village during last month:	70 (11.2%)	910 (22.8%)	64 (10.6%)
Have bednets:	608 (97.6%)	3867 (96.7%)	578 (95.7%)
Reported use of bednets:			
Every night	539 (86.5%)	3351 (86.8%)	510 (84.4%)
Sometimes	67 (10.8%)	484 (12.1%)	61 (10.1%)
Not used	2 (0.3%)	32 (0.8%)	7 (1.2%)
No net	15 (2.4%)	133 (3.3%)	26 (4.3%)

* n (%)

The distribution of infection in relation to several demographic variables is shown in Table 3.10. The prevalence of slide positivity among males and females was significantly different. It is probable that males had more chance to contact mosquitoes due to their jobs causing them to stay temporarily in the forest zone for plantation or forest related activities, such as nights spent guarding their crops in the fields. Another factor was that women often spend the hours around dusk preparing food in the smoky environment of the kitchen, which probably is repellent to mosquitoes. The prevalence of malaria infections appeared slightly higher in the Karen ethnic group but there was no significant difference between ethnic groups. A highly significant relationship between occupation and percent positive was observed, with an apparently higher prevalence in the school student group.

Table 3.10 Distribution of infection in relation to demographic variables, with chi-squared tests between slide positives and slide negatives for each variable.

	Slides		Significance test	*P* value
	Total interviewed	**No. positive (%)**		
Sex:				
Male	2469	35 (1.4%)	χ^2 with Yates' correction = 6.92 $df = 1$	$P = 0.008$
Female	2760	18 (0.7%)		
Ethnicity:				
Thai Yai	3174	28 (1.0%)	χ^2 with Yates' correction = 4.48 $df = 2$	$P = 0.11$
Karen	1240	19 (1.5%)		
Other	813	6 (0.9%)		
Occupation:				
School students	673	16 (2.4%)	χ^2 with Yates' correction = 18.08 $df = 3$	$P < 0.001$
Farmer	2663	21 (0.8%)		
Wage earner	1315	15 (1.1%)		
Housewives & other	576	1 (0.2%)		

3.1.2.1 Behaviour in relation to malaria

3.1.2.1.1 Recalled malaria episodes

Responses to a question about the months in which the interviewee recalled having any malaria episode are shown in Figure 3.4. The total number of most recent malaria episodes recalled was 296 episodes for 1999 and 123 for January to August 2000. The highest percentage of reports was for the month of May in both years (19.9% in 1999 and 37.4% in 2000). This agrees with the reported incidence of clinically diagnosed malaria for Thailand (see Figure 1.16 in chapter 1).

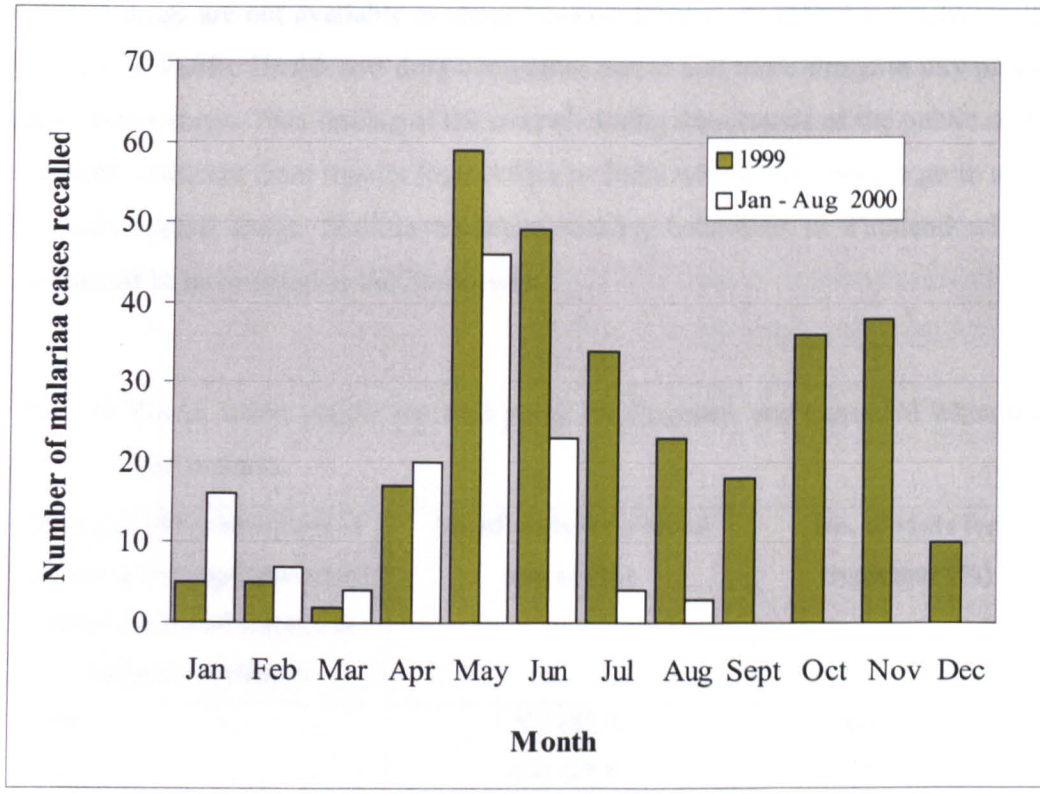

Figure 3.4 Month of most recent malaria episode recalled in 1999 and 2000 (data until August 2000 only).

3.1.2.1.2 Treatment-seeking behaviour

A question was asked about where people went to have their blood smear checked and to seek malaria treatment during the previous three years (i.e. during 1998-2000). It was found that the Vector-borne Disease Control Unit no.8 (VBDU: described as "Clinic" throughout this thesis) and the hospital facilities were most commonly used (Table 3.11) with the largest number going to the Clinic when they thought they had malaria. This validates the distinction made in the work in chapter 4 where feeds on Clinic patients are taken as representing those who felt ill with malaria. Surprisingly, only 4 cases gave the answer that they went to see a Village Malaria Volunteer (VMV) for blood smears. VMV's do not seem to be attractive for the people. The facilities at which anti-malarial drugs were sought were similar to the places which people chose for having blood smears checked. In Thailand, anti-

malarial drugs are not available in shops because there is an agreement between the Ministry of Public Health and drug companies not to sell these drugs to any private providers or shops. This finding of the overwhelming importance of the public sector is entirely different from reports from Africa or India where most people go to shops for anti-malarial drugs. Malaria treatment-seeking behaviour in Thailand will be considered in more detail in the Discussion.

Table 3.11 Places where people reported going for diagnosis and treatment when they had malaria.

Places at which members of the surveyed population had visited for blood smears or malaria treatment	**No. of visits for a blood smear (%)**	**No. of visits for treatment (%)**
Clinic	555 (45.9)	648 (53.7)
Hospital	360 (29.8)	383 (31.7)
Mobile malaria clinic	187 (15.5)	80 (6.6)
Health centre	92 (7.6)	79 (6.5)
Private clinic	6 (0.5)	8 (0.7)
Refugee camp health centre	4 (0.3)	4 (0.3)
Village Malaria Volunteer	4 (0.3)	1 (0.1)
Dispensary	0 (0)	4 (0.3)
Traditional doctor	0 (0)	1 (0.1)
Total	1208 (100)	1208 (100)

3.1.2.1.3 Follow-up after treatment

Among the village survey interviewees who reported having been to the Clinic a question was asked about whether they had been back for a follow-up blood slide. The response was related to our observation of slide positivity or negativity at the time of the surveys. It was found that less than 50% of people who had been diagnosed and treated at the Clinic for malarial infections during 1998-2000, reported having gone back for a blood check. Almost equal proportions still with

malaria parasites were found among those who had gone voluntarily for a re-check and those who had not (Table 3.12). This finding was similar to the situation at the Clinic (personal communication from Clinic staff). Some patients stopped taking anti-malarial drugs after they recovered from a fever or when a serious side effect from the drugs was encountered and did not come back for a blood test. Some live far away from the clinic and would have to pay for transport back to the Clinic, and if they needed to take one day off, it would mean that they would lose their income for that day as well. This presumably explains why people are reluctant to go back for a re-check even though the Clinic staff emphasise the importance of such a follow up visit, especially because of the risk of failure of anti-malarial drugs due to resistance.

Table 3.12 Prevalence of malaria found in our surveys among those who reported in the village surveys to have been to the malaria clinic, related to whether they reported going for a follow-up check.

Follow up	No. positive for malaria (%)	Total number
No	13 (2.1)	632
Yes	12 (2.1)	576
Total	25 (2.1)	1208

3.1.2.1.4 Relationship of staying outside village and malaria episodes

People with a history of staying outside their village had a much higher prevalence of infection than those people who had not stayed outside their villages at night. 80% of the surveyed population had not stayed outside their village during the previous month. Among those, 25 positive cases (0.6%) were found (Table 3.13). People who had stayed outside their villages for more than 6 nights in the last month showed 4.0% slide positivity (18/453). There was a highly significant association between the prevalence and the number of nights spent outside the village in the last month (χ^2 for linear trend = 48.05, P <0.001). People who stay outside their villages

mostly live in temporary huts with incomplete walls which are easily entered by mosquitoes such as *An. minimus* A which is the main vector in this area (Ratanatham *et al.*, 1988; Somboon *et al.*, 1998). Moreover, most of those who travel say that they do not take mosquito nets with them but sleep in huts or temporary houses in forests without nets. This exposes them to a high risk of infection with malaria. When they returned to their villages on the forest fringes they frequently become malaria cases or asymptomatic carriers and may become an important reservoir of infection of the village mosquito populations (see Table 4.9 in chapter 4).

Table 3.13 Number of nights that people had stayed outside their village in the last month and proportion positive for malaria infection.

Nights	Total slides	No. positive for malaria (%)
0	4183	25 (0.6)
1	80	1 (1.3)
2	208	2 (1.0)
3	154	4 (2.6)
4	53	2 (3.8)
5	96	1 (1.0)
6+	453	18 (4.0)
Total	5227	53 (1.0)

3.1.2.1.5 Mosquito protection methods

Only 45 interviewees reported that they did not use any methods of mosquito protection and 99.6% (5053/5227) owned mosquito nets. Reported bednet usage was 99.2% (5012/5053) and this was by far the most common preventive measure reported to be used. According to Table 3.14, 84.2% (4400/5227) reported using nets every night, 11.7% (612/5227) used them irregularly and a few never used them. Approximately 44% of all the nets had been treated with insecticide at some time in their lives. Some villages, had been provided with free treatment for nets by the national government using permethrin 10% EC under the "Net Treatment

Programme", but 1,074 out of 3,300 (32.5%) of people in these villages had not treated their nets. Very few reported that they used smoky fires or window screening to repel mosquitoes. The results showed that the prevalence of infection in this small group (1.6%) was significantly lower than in the group who reported using no protection (8.9%).

The results showed that 0.9% (44/5012) of people who had bednets (treated or untreated) and used them were positive, whilst 7.3% (3/41) who had nets but stated that they did not use them were infected. This difference was significant (Fisher's exact test, $P < 0.05$) and there was no significant difference between the prevalence in the latter group and those who reported not having any form of mosquito protection. The villagers had an average of 1.5 mosquito nets per family and an average of 2 persons shared each net. This finding is similar to the 1999 annual report of the Malaria Division, of the Thai Ministry of Public Health, which reported 2.3 persons/net and to the study of Butraporn *et al.* (1995) which indicated that approximately 2-3 persons shared a net. Among those without nets, the prevalence of parasite positivity was approximately three times higher than in those owning nets, the difference being statistically significant ($P = 0.01$). The findings emphasise that the use of mosquito protection methods has a very beneficial impact in reducing malaria infections.

Table 3.14 shows other statistical comparisons. A significant difference in prevalence of malaria was observed between users of treated and untreated nets in treated villages and also with untreated nets in untreated villages. However, there was no significant difference between those with untreated nets in villages with predominantly treated nets and those without them. These results indicate that the net treatment is providing personal protection but that widespread use of treated nets is not providing additional community protection by its impact on the vector population. The group without mosquito protection was significantly different from all other groups except those who had nets but did not use them (Table 3.13). Possible reasons why some people do not treat their nets and did not have this added protection will be considered in the Discussion.

Table 3.14 Prevalence of malaria infections in people using treated/or untreated nets or no nets.

	No. positive cases/total slides (% prevalence)	**Statistical test (categories compared)**
A. Treated nets	10/2226 (0.5%)	(A & B) χ^2 with Yates' correction = 5.02, $P = 0.02$ (A & C) χ^2 with Yates' correction = 6.53, $P = 0.01$
B. Untreated nets in treated villages	13/1074 (1.2%)	(B & C) χ^2 with Yates' correction = 0.02, $P = 0.89$
C. Untreated nets in untreated villages	21/1712 (1.2%)	(C & D) Fisher exact test, $P = 0.01$
D. Nets owned but not used	3/41 (7.3%)	(D & F) Fisher's exact test, $P = 1.0$
E. Other mosquito protection*	2/129 (1.6%)	(E & F) Fisher's exact test, $P = 0.03$
F. No mosquito protection	4/45 (8.9%)	Fisher's exact test (F & A) $P < 0.001$, (F & B) $P = 0.003$ (F & C) $P = 0.003$,

* Smoky fires or window screening

3.1.3 Prevalence of symptomatic and asymptomatic infections

Table 3.15 shows the results associated with 5,227 blood smears from village surveys. 10.7% (561/5,227) of subjects reported fever but the corresponding rate for slide positives was 52.8% (28/53). Conversely, 4.9% (28/561) of those reporting fever were slide positive, and about half of these had a parasite density > 3,960/μl (this is equivalent to >99 /200 WBC). Of the 89.3% who did not report fever, only 0.5 % were slide positive and a minority of these had a parasite density >3,960 parasite/μl. There was a strong association of measured body temperature and slide positivity (χ^2 for linear trend = 7.87, P <0.001). Among people having a body

temperature >37.4°C 9/115 (7.8%) were slide positive, as compared to those with a body temperature <37.5°C where the proportion was 44/5112 (0.8%). 25/53 (47.1%) of slide positive cases were asymptomatic, i.e. with neither a report of fever nor temperature >37.4°C. Among those, 21/25 had a parasite density less than 3,960 parasites/μl whereas only 13/28 of symptomatic cases had low parasitaemia (Table 3.15).

Table 3.15 Relationship of parasite density, reported fever and measured temperature from village surveys

	Slide negative (%)	**Parasite density 40-3960/μl (%)**	**Parasite density >3960/μl (%)**	**Total (%)**
Reported fever in previous week:				
Yes	533 (94.84)	13 (2.3)	15 (2.7)	561 (10.7)
No	4641 (99.40)	21 (0.5)	4 (0.1)	4666 (89.3)
Temperature:				
<37.5°C	5068 (99.06)	31 (0.6)	13 (0.3)	5112 (97.8)
37.5-38.5°C	92 (94.85)	3 (3.1)	2 (2.1)	97 (1.9)
>38.5°C	14 (77.78)	0 (0)	4 (22.2)	18 (0.3)
Symptomatic:				
Yes*	592 (95.33)	13 (2.3)	15 (2.4)	620 (11.9)
No	4582 (99.39)	21 (0.5)	4 (0.1)	4607 (88.1)

* body temperature >37.4°C and/or reported fever

As shown in Table 3.16, among patients with positive slides who reported fever, some had a measured temperature <37.5°C. However, all those with positive slides who had a temperature >37.4°C also reported fever, i.e. the original definition of "fever" as a recorded temperature > 37.4°C and/or reported fever gave equivalent results to a definition of fever as "reported fever". There was no significant difference of prevalence of fever between *P. falciparum* and *P. vivax* infections

(Yates' correction = 0.63, P = 0.42). The average body temperature of those with positive slides was 36.3°C.

Table 3.16 Body temperature (measured using an ear thermometer) in those reporting, and those not reporting fever among patients with positive slides in the village surveys.

Body temperature	**No reported fever (%)**	**Reported fever (%)**	**Total positive slides**
<37.5°C	25 (56.8)	19 (43.2)	44
37.5-38.5°C	0	5 (100)	5
>38.5°C	0	4 (100)	4
Total	25	28	53

As shown in Table 3.17, among 25 cases of asymptomatic malaria infection 13 were infected with *P. falciparum*, 11 with *P. vivax* and 1 was a mixed infection. The total number of infections identified were 24 for *P. falciparum* and 28 for *P. vivax*. Thus the probability of an infection being asymptomatic appeared to be slightly higher for *P. falciparum* than *P. vivax* but the difference was not significant (χ^2 with Yates' correction = 0.63, P = 0.43). Only five gametocyte carriers were asymptomatic (2 cases in children and 3 in adults). The observed parasite densities were slightly higher in adults than in children but no significant difference was found ($t = 1.36$, $df = 25$, $P = 0.18$). There were more asymptomatic cases among males than females but there were also more infections in males (Table 3.6) and there was no association of sex with tendency of an infection to be asymptomatic (χ^2 with Yates' correction = 0.33, P = 0.56).

Table 3.17 Asymptomatic parasitaemia from the village surveys by age group with percentage of infections with each species that were asymptomatic.

Characteristics	Age groups			Total
	5-14	15-59	60+	
Total number slide positive	16	34	3	53
Asymptomatic	7 (43.6%)	16 (47.1%)	2 (66.7%)	25 (47.2%)
Sex:				
Male	4 (51.1%)	13 (81.3%)	1 (50.0%)	18
Female	3 (48.9%)	3 (18.8%)	1 (50.0%)	7
No. *P. falciparum* +ve	5	18	1	24
Asymptomatic	3 (60%)	10 (55.6%)	0	13 (54.2%)
Parasite density/μl in asymptomatic cases	261.6	1578	0	1042.4
No. *P. vivax* +ve	10	16	2	28
Asymptomatic	3 (30.0%)	6 (37.5%)	2 (100%)	11 (39.3%)
Parasite density/μl in asymptomatic cases	1,316.8	1502.8	1443.2	1,439.2
Mixed infection	1	0	0	1
Asymptomatic	1 (100%)	0	0	1 (100%)
Parasite density/μl in asymptomatic cases	200			200

3.2 Malaria Clinic

3.2.1 Presence of fever and parasitaemia

From the Clinic, 101 patients with malaria infections were interviewed, 85.2% of the patients reported fever and about half of these had a temperature >37.4°C. The geometric mean parasite densities were 4,996/μl (95% CI = 3,206.2-7,788.3) for *P. falciparum* and 3,570.8/μl (95% CI =2,489-5,123.1) for *P. vivax*; the difference was not significant (t = 1.18, *df* = 99, *P* = 0.23). The geometric mean

gametocyte densities were lower for *P. falciparum* (94/μl; 95% CI = 53.7-164.8) than *P. vivax* (350.8/μl; 95%CI = 234.7-524.9) and the difference was significant (t = 3.44, df = 48, P = 0.001). More than half of the infections detected at the Clinic had parasite density >3,960/μl (Table 3.18). 79.6% of *P. falciparum* and 90.4% of *P. vivax* infections were associated with reported fever.

As shown in Table 3.19 more than half of the cases from the Clinic who had fever in the previous week and a temperature >37.4°C, had a high parasitaemia of either *P. falciparum* or *P. vivax*. Among those with reported fever there was no association between parasite densities and the measured body temperature when the parasite species were combined (χ^2 = 1.16, df = 2, P = 0.56). Presumably having a fever was the main motivation for people to come to the Clinic and it is not clear why some patients who came to the Clinic said that they did not have fever. However, about 14.8% (15/101) of those with positive slides did give this answer to the questionnaire. None had a measured temperature above 37.4°C and most had low parasitaemia. Approximately 44.4% (20/45) in the low parasitaemia group had gametocytaemia and 53.6% (30/56) had gametocytaemia in the high parasitaemia group.

Table 3.18 Relationship of parasite density, reported fever and measured temperature in cases positive for parasitaemia from the Clinic.

Parasite species		Parasite density 40-3960/μl (%)	Parasite density >3960/μl (%)	Total
	Reported fever &:			
	Temp. <37.5°C	6 (27.3%) (g=1)	16 (72.7%) (g=4)	22 (g=5)
	Temp. 37.5-38.5°C	5 (55.6%)	4 (44.4%)	9 (g=0)
	Temp. >38.5°C	2 (25.0%)	6 (75.0%) (g=2)	8 (g=2)
P. falciparum	No Reported fever:			
(n = 49)	&			
	Temp. <37.5°C	7 (70.0%) (g=3)	3 (30.0%) (g=2)	10 (g=5)
	Temp. 37.5-38.5°C	0	0	0
	Temp. >38.5°C	0	0	0
	Reported fever &:			
	Temp. <37.5°C	9 (45.0%) (g=7)	11 (55.0%) (g=9)	20 (g=16)
	Temp. 37.5-38.5°C	5 (45.45%) (g=4)	6 (54.55%) (g=5)	11 (g=9)
P. vivax	Temp. >38.5°C	8 (50.0%) (g=4)	8 (50.0%) (g=6)	16 (g=10)
(n = 52)	No Reported fever			
	&:			
	Temp. <37.5°C	3 (60.0%) (g=1)	2 (40.0%) (g=2)	5 (g=3)
	Temp. 37.5-38.5°C	0	0	0
	Temp. >38.5°C	0	0	0
Total		45 (44.6%) (g=20)	56 (55.4%) (g=30)	101(g=50)

Letter "g" in the brackets refers to number of cases with gametocytes

Table 3.19 Relationship of parasite density, reported fever in the previous week and measured temperature in cases positive for parasitaemia from the Clinic, with parasite species combined (in each fever category percentage with low or high parasitaemia are indicated in parentheses).

	Parasite density 40-3960/μl (%)	**Parasite density >3960/μl (%)**	**Total**
Reported fever &:			
Temp. <37.5°C	15 (35.7%) (g=8)	27 (64.3%) (g=13)	42 (g=21)
Temp. 37.5-38.5°C	10 (50.0%) (g=4)	10 (50.0%) (g=5)	20 (g=9)
Temp. >38.5°C	10 (41.7%) (g=4)	14 (58.3%) (g=8)	24 (g=12)
No Reported fever &:			
Temp. <37.5°C	10 (66.7%) (g=4)	5 (33.3%) (g=4)	15 (g=8)
Temp. 37.5-38.5°C	0	0	0
Temp. >38.5°C	0	0	0
	45 (44.6%) (g=20)	56 (55.5%) (g=30)	101 (g=50)

Letter "g" in the brackets refers to number of cases with gametocytes

The proportion of cases from the Clinic with reported fever (85.2%) was higher than from the village surveys (52.8% of slide positives). However, the percentage of high parasite density (>3,960/μl) from those who reported fever was similar in both samples (52.8% from village survey and 59.3% from the Clinic). The proportion of infections that were asymptomatic was higher in the village surveys [45.3% (25/53) of slide positives, table 3.15] than in the Clinic [14.9% (15/101); table 3.19]. This is to be expected since in most cases people were presumably motivated to go to the Clinic by feeling malaria symptoms. There was a strong association of measured body temperature with a high parasite counts from village surveys (Table 3.15) but not from the Clinic (Table 3.19).

3.3 Discussion

This part of the study has provided data from villages in three cantons on parasite rates, gametocyte rates, prevalence of asymptomatic infections, treatment seeking behaviour and the impact of mosquito protection methods.

The parasite prevalence in the village surveys was 0.7% (57/7,812). This is lower than routinely reported by the Malaria Clinic. Prior to the performance of the current survey, a policy of 100% coverage by active case detection was adopted by the Provincial Government in highly malarious areas including this province. 10 villages in the present study were included in the category of high prevalence villages for intensive routine surveys. This active case detection is carried out at least twice a month and tries to cover 100% of the population. Thus the prevalence of malaria infections that were detected in the present study are presumably considerably reduced as a result of the treatments which follow this provincially organised active case detection campaign, especially with regard to those asymptomatic for malaria. The prevalence of malaria infection was similar in children and adults (0.7% vs. 0.8%) (Table 3.7). This finding contrasts with those in the highly endemic areas of Africa where higher rates of *P. falciparum* and *P. malariae* are found in the younger age groups (e.g. Imperato, 1986) so that the highest prevalence is in children under five years of age and the infection rates decrease with age. However, several studies in Africa indicated that parasite prevalence is lower in the first year of life than in subsequent years and is especially low in children less than 3-4 months old (Hogh, 1991; Akanmori et al., 1995; Snow et al., 1996). In Africa parasite and spleen rates normally reach a peak in children under five years of age and decline substantially by the age of 15, indicating increasing protection due to acquired immunity (Wilson, 1950; Williamson and Gilles, 1978; Rosenberg and Maheswary, 1982a). The situation is different for Thailand where acquired immunity does not commonly occur in children under five years, but it may develop in adults. A few studies have reported such naturally acquired immunity in areas endemic for *P. vivax* (Gamage Mendis *et al.*, 1991; Luxemburger *et al.*, 1999).

In the present study the prevalence of malaria was significantly higher in men than women (Table 3.10). The possible reasons have been discussed in section 3.1.2.1. However, results from several other studies showed that the ratio of malaria prevalence between men and women varies from place to place and from one year to another, depending on many interrelated factors.

In Thailand, *P. falciparum* continued to be found as the predominant species for more than 50 years. However, in the national data from the year 2000, *P. vivax* for the first time surpassed *P. falciparum* (Figure 1.10). In the present study *P. vivax* accounted for 56.1% of species identifications, *P. falciparum* was observed in 42.1% and mixed infection was found in 1.8% (Table 3.7).

The present study showed that all those with positive slides who had a body temperature >37.4°C reported fever. Some without measured body temperature >37.4°C also reported fever. Those people may have had fever a few hours ago. There was no significant difference in prevalence of fever between *P. falciparum* and *P. vivax* infections. 0.5% of all blood smears, or 47.2% of slide positives, were aymptomatic malaria cases (Table 3.17). This prevalence of asymptomatic cases was lower than a report from eastern Thailand where 66-77% of malarial infections (9.31-12.6% of the surveyed population) (Kamol-Ratanakul *et al.*, 1992; 1994) were asymptomatic. Similarly from the western border of Thailand 59% of school children in whom *P. vivax* was detected were asymptomatic (Luxemburger *et al.*, 1999). However, even in that study the proportion of asymptomatic cases was quite low when compared with high transmission areas such as Papua New Guinea (Cattani *et al.*, 1986). Asymptomatic carriers may play an important role as a reservoir maintaining parasites in communities, especially in high transmission areas of developing countries where equipment or skilled staff are lacking and it is difficult to detect such cases by microscopy, especially when densities of parasites are very low.

The questionnaire showed decisively that when people think they have malaria the Clinic or hospital were the common places where the great majority sought help (Table 3.11). As mentioned in section 3.1.2.1.2 anti-malarial drugs are not available through the private sector, thus home care or self-medication for a malaria infection is now not common in Thailand. However, in Thailand the availability of anti-malarial drugs such as chloroquine was widespread in village groceries in the past. Kamol-Ratanakul *et al.* (1992) reported that in the 1980s people in rural areas in eastern Thailand usually self medicated when they experienced malaria attacks by using a drug preparation under the name of "ya-chud" bought from village groceries. However, in some countries near to Thailand, such as Vietnam and Cambodia, people are able to buy anti-malarial drugs from shops. It is reported from Vietnam that there are few drugs which remain effective and are available at health posts but certain new drugs (artemisinin, artisunate and mefloquine) have become available through the private sector but not at government clinics. Thus some patients, who can afford the new drugs, prefer to go to the private sector (Van Kim, 1999). There is widespread treatment of malaria in Africa and India at home with herbal remedies or medicines purchased at local shops (Foster, 1991; Marsh *et al.*, 1995; Ruebush *et al.*, 1995; Nyamongo, 2002). This almost certainly leads to delays in seeking proper treatment in cases of treatment failure due to sulfadoxine-pyrimethamine resistance. Such improper use of anti-malarial drugs has recently caused a major increase in malaria mortality in Africa (Trape *et al.*, 2002) and has presumably increased the rate of evolution of drug resistance.

The answers to the questionnaire about the use of mosquito protection methods showed that 99.6% of the surveyed population owned mosquito nets but only 44% of nets had been treated with a pyrethroid at some time in their existence (Chapter 3, page 127). Approximately 40% of people in the study area did not treat their nets because they perceived a smell of insecticide and malaria staff do not treat nets for pregnant women (personal communication from the malaria staff at the Clinic). People who used treated nets experienced lower prevalence of malaria infections than those who slept under untreated nets (Table 3.14). Numerous intervention trials in the 1990s have shown the value of treated nets (Lengeler,

1998). More recently Schellenberg *et al.* (2001) have reported on increase in survival up to 27% in children aged 1 month to 4 years among treated net users in rural Tanzania. Similar results have been shown for children in refugee camps on the Thai-Myanmar border (Luxemburger *et al.*, 1994). However, apart from the data in Table 3.14 there are relatively few reports of such effects from observations on nets in routine use. Although, impregnated bednets have a high potential efficacy against malaria, lack of careful use may prevent the realisation of their potential. In the present study 84.2% reported using bednets regularly, whereas 11.7% used them infrequently and a few never used them (section 3.1.2.1.5). In a study in northern Thailand it was found that about 82% of households in the villages owned bednets but only 70% of villagers working outside the villages carried them to the forest. Some people did not consider it worthwhile to sleep under nets (Chitprarop, 1986). This proportion was higher than in a study from the same region by Aramrattana (1993), who found that for only 52.3% of the total person-nights away from home had nets been brought. Butraporn *et al.* (1995) reported from a study in eastern Thailand that only 34.0% brought their nets with them when travelling for an overnight stay outside their villages. Somboon *et al.* (1998) considered that biting of vectors generally occurred at farm huts near forests and the relative risk of infection for people involved in agricultural activity was three times higher than those permanently residing in villages. An increased exposure to malaria vectors can be expected among those migrants who do not bring their nets with them when they stay out overnight in areas where other humans visit so that previous infection of the mosquitoes could have occurred. However, in remote forest areas it seems unlikely that one would encounter a vector which had previously bitten a human and acquired human malaria infection.

Data exist demonstrating the mass effect of widespread use of treated nets in Tanzania (Magesa *et al.*, 1991; Curtis *et al.*, 1998; Maxwell *et al.*, 1999). These showed that community-wide use of treated nets reduced vector survival and therefore vectorial capacity. These results are consistent with studies from Sierra Leone (Magbity *et al.*, 1997), Ghana (Binka *et al.*, 1996), and Cameroon (Le Goff *et al.*, 1993). In contrast no such mass effects of treated nets were reported from Kenya

(Mbogo *et al.*, 1995), The Gambia (Quiñones *et al.*, 1998) or Thailand (Prasittisuk *et al.*, 1992; Somboon *et al.*, 1995). However, the efficacy of treated nets in reducing malaria incidence in those communities was found. Similarly, in the present study (Table 3.14) no difference was found between those not using treated nets in villages when others were using them, in comparison to villages with no treated nets. This contrasts with data of Maxwell *et al.* (submitted) from Tanzania where such differences were clear.

In conclusion, the present study shows that malaria infection is found in this part of Thailand about equally in children and adults. Gametocytes occurred about equally in adults and children as in other areas where malaria transmission is unstable. The results of the present study demonstrate approximately the same prevalence of asymptomatic malaria as seen in previous studies in Thailand. The probability of an infection being asymptomatic was similar in *P. falciparum* and *P. vivax* (Table 3.17). This finding was different from a report of Luxemburger *et al.* (1999) that patent *P. vivax* parasitaemias were more likely to be asymptomatic than those with *P. falciparum.* Since several studies have suggested that asymptomatic malaria may play an important role in maintaining transmission in communities, the infectiousness of symptomatic and asymptomatic cases to mosquitoes have been compared, as described in the next chapter.

CHAPTER 4

EXPERIMENTS ON MOSQUITO INFECTION

4.1 General characteristics of the human subjects

4.1.1 Demographic data

130 *Plasmodium* infected human subjects with or without gametocytes were enrolled in the mosquito feeding study (29 cases from village surveys and 101 cases from the Clinic). No mosquito feedings were done on children less than 15 years of age as the Thai ethnical committee did not allow this. Thus there is a lack information about the importance of children as a reservoir for infection of mosquitoes. The predominant ethnic group was Thai Yai (70%).

From positive cases found in the village surveys, 29 cases were used in the feeding experiments and among those 12 (41.4%) cases were asymptomatic. 14 of the cases were *P. falciparum* and 15 were *P. vivax*. 101 malarial infections were located via the Clinic; 49 of these were *P. falciparum* and 52 were *P. vivax*. Only 15% of the cases from the Clinic were reported to be asymptomatic.

Cases were only included in the analysis when 5 or more of the fed mosquitoes were dissected. Thus data on 28 cases from village surveys and 92 cases from the Clinic were analysed.

Table 4.1 shows the characteristics of infected human subjects from village surveys and the Clinic. More males than females were used in the feeding experiments both from the village surveys and the Clinic but there was no significant difference between the sex ratios used from the villages and the Clinic. The observed mean age was higher from the Clinic but the difference was not significant. There was a significant difference in mean body temperature between the cases used from village surveys and the Clinic ($t = 2.57$, $df = 118$, $P = 0.01$). This is not surprising as the Clinic sample was self-selected as fever cases who decided to travel to the Clinic.

Table 4.1 Characteristics of malaria cases located in village surveys and the Clinic on which mosquitoes were fed.

Characteristics	Village surveys	Clinic	Significance tests between village surveys and the Clinic
No. used for analysis	28	92	
Sex			
Male	23 (82.1%)	71 (77.2%)	$\chi^2 = 0.09$, $P = 0.76$
Female	5 (17.9%)	21 (22.8%)	
Mean age (years)	25.5	28.9	$t = 1.37$, $df = 118$, $P = 0.17$
95% CI	21.5-30.3	26.5-31.5	
Mean body temperature	36.8	37.5	$t = 2.57$, $df = 118$, $P = 0.01$
95% CI	36.5-37.1	37.2-37.8	

4.1.2 Parasitology indices

Among 120 cases used for the mosquito experiments, the geometric mean of asexual parasite densities was higher for *P. falciparum* than for *P. vivax* but the difference was not significant ($t = 1.20$, $df = 118$, $P = 0.23$) while the mean gametocyte counts were considerably higher in *P. vivax*, the means being significantly different between the species ($t = 2.82$, $df = 56$, $P = 0.006$).

The geometric mean asexual parasite density was slightly but not significantly higher in the *P. falciparum* sample from the Clinic than from the village surveys but the difference was significant for *P. vivax*. The mean gametocyte count did not differ significantly between the village surveys and the Clinic (Table 4.2).

58 of the cases used in the mosquito experiments had observable gametocytes (11 cases from the village survey and 47 from the Clinic). The distribution of gametocyte densities in cases from the village surveys and the Clinic are presented in Figure 4.1. More than 80% of the cases from the village surveys had gametocyte densities ≤ 800/μl (range 40 to 8,400/μl) and the corresponding figures were 70%

(range 80 to 3,920/μl) from the Clinic. The mean age of gametocyte carriers was 31.6 years (range = 15-70).

Table 4.2 Geometric mean of parasite densities of the two parasite species from the cases used for the mosquito feeding experiments.

Characteristics	**Village surveys**	**Clinic**	**Statistical test**
No. used for analysis	28	92	
No. of *P. falciparum* (%)	13 (46.4%)	44 (47.8%)	
Mean asexual parasites/μl	4,384	4,608	$t = 0.09$, $df = 55$, $P = 0.92$
Range	200-60,280	280-72,080	
Mean gametocyte density/μl	224	100	$t = 1.18$, $df = 11$, $P = 0.26$
Range	80-1,200	40-400	
No. of *P. vivax* (%)	15 (53.6%)	48 (52.2%)	
Mean asexual parasites/μl	1849	3908	$t = 1.85$, $df = 61$, $P = 0.06$
Range	40-18,320	80-68,640	
Mean gametocyte density/μl	360	356	$t = 0.02$, $df = 43$, $P = 0.97$
Range	40-3,920	40-8,400	

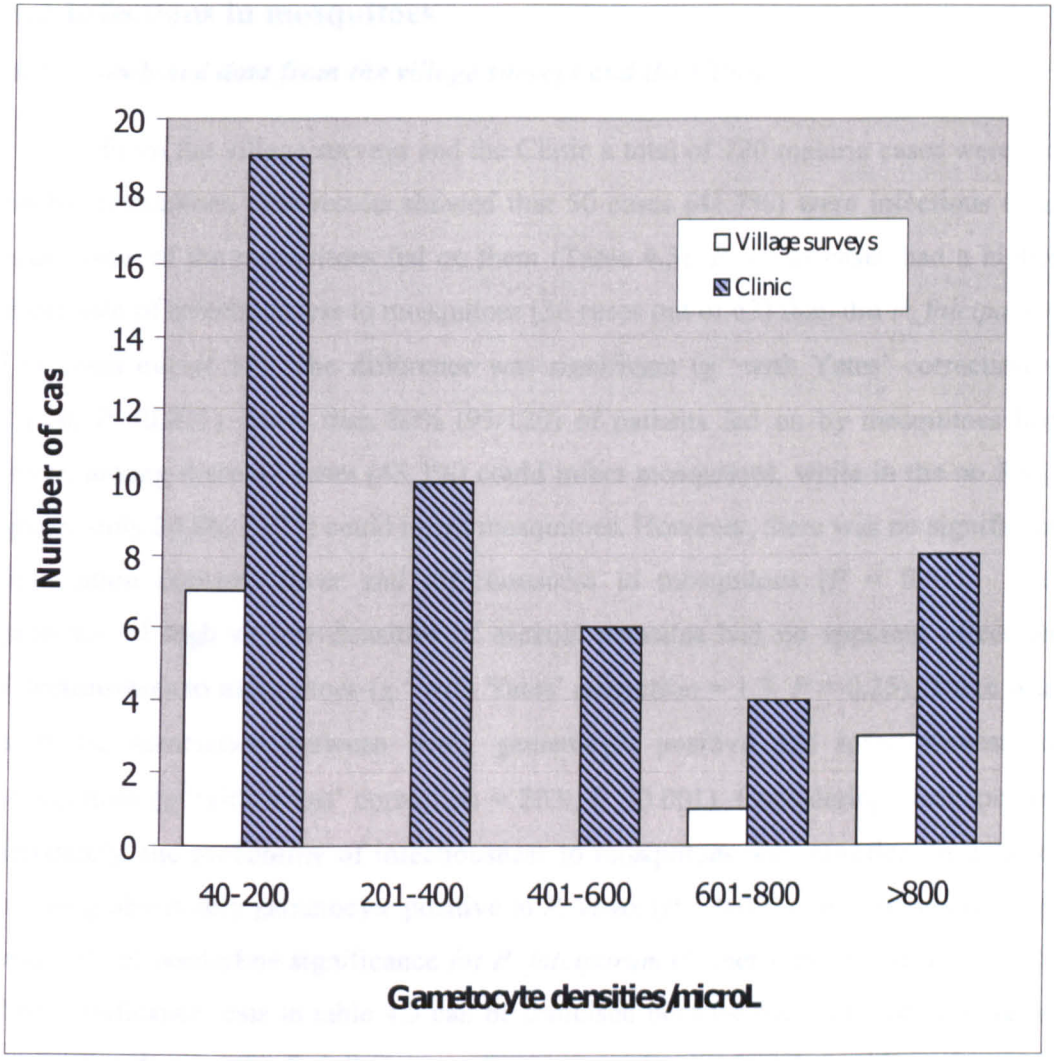

Figure 4.1 Frequency distribution of gametocyte densities among 11 gametocyte carriers from village surveys and 47 from the Clinic, combined species.

4.2 Infections in mosquitoes

4.2.1 Combined data from the village surveys and the Clinic

From the village surveys and the Clinic a total of 120 malaria cases were fed on by mosquitoes. The results showed that 50 cases (41.7%) were infectious to at least some of the mosquitoes fed on them (Table 4.3). *P. vivax* cases had a higher mean rate of infectiousness to mosquitoes (36 cases out of 63) than did *P. falciparum* (14 cases out of 57). The difference was significant (χ^2 with Yates' correction = 11.76, P <0.001). More than 80% (97/120) of patients fed on by mosquitoes had fever; among these 42 cases (43.3%) could infect mosquitoes, while in the no fever group, only 34.8% (8/23) could infect mosquitoes. However, there was no significant association between fever and infectiousness to mosquitoes (P = 0.61). The presence of high or low densities of asexual parasites had no apparent effect on infectiousness to mosquitoes (χ^2 with Yates' correction = 1.3, P = 0.25). There was a strong association between being gametocyte positive and infectiousness to mosquitoes (χ^2 with Yates' correction = 20.9, P <0.001). Considering each species separately, the probability of infectiousness to mosquitoes was significantly related to being observably gametocyte positive in *P. vivax* (P = 0.007), but the relationship was only of borderline significance for *P. falciparum* (Fisher's exact test, P = 0.06). The significance tests in table 4.3 can be criticised because they involve pooling of categories despite the fact that some factors may be confounded with each other. Therefore table 4.4 cross-tabulates the data on infectiousness to mosquitoes into cases with *P. falciparum* and *P. vivax*, high/low parasite densities, presence of observable gametocytes and presence of fever. 84.1% (53/63) of *P. vivax* cases had body temperature >37.4°C and/or reported fever. Among those, 75.5% (40/53) had gametocytaemia and 67.5% (27/40) of cases with observable gametocytaemia could infect mosquitoes. For *P. falciparum* 77.2% (44/57) had body temperature >37.4°C and/or reported fever, among those 22.7% (10/44) had gametocytaemia and 50% (5/10) of the cases with observable gametocytaemia could infect mosquitoes. That is approximately 50% of the *P. falciparum* cases with observable gametocytes failed to infect mosquitoes and 32% of the *P. vivax* cases with observable gametocytes failed to infect. However, the difference was not significant (Fisher's exact test, P = 0.19).

In the group with no fever, a few cases had observable gametocytes and could infect mosquitoes. Mantel-Haenszel (MH) χ^2 tests were used on the data in table 4.4 stratified by fever, species, and presence of gametocytes and/or parasite density (Table 4.5). These tests confirm the conclusions from table 4.3 that there was no association of probability of mosquito infection with presence of fever or with parasite density. They also confirm the conclusion that (as might be expected) there was association of probability of mosquito infection with presence of observable gametocytes. However, table 4.5 does not confirm that there was a significant difference between parasite species.

Table 4.3 Number of malaria infected people who infected with oocysts at least some of the mosquitoes fed on them. Cases classified by (a) parasite species (b) presence of fever, (c) low or high parasite densities and (see section 3.1.1.2) (d) presence of observable gametocytes. Category (d) is further split into . *falciparum* and *P. vivax*. Cases from the village surveys and the Clinic are combined in this table.

Characteristics	**Oocyst +ve*** (n = 50)**	**Oocyst –ve (n = 70)**	**Significance tests**
No. positive/negative for oocyst production by any of the mosquitoes feed on each person	50/120 (41.7%)	70/120 (58.3%)	
(a) Parasite species:			
P. falciparum	14 (24.6%)	43 (75.4%)	χ^2 with Yates' correction
P. vivax	36 (57.1%)	27 (42.9%)	= 11.76, P <0.001
(b) Fever:			
Yes*	42 (43.3%)	55 (56.7%)	χ^2 with Yates' correction
No	8 (34.8%)	15 (65.2%)	= 0.26, P = 0.61
(c) Parasite densities:			
Low (40-3,960/µl)	21 (35.59%)	38 (64.41%)	χ^2 with Yates' correction
High (>3,960/µl)	29 (47.54%)	32 (52.46%)	=1.30, P = 0.25
(d) Observable gametocytes:			
Yes	37 (63.8%)	21 (36.2%)	χ^2 with Yates' correction
No **	13 (21%)	49 (79%)	= 20.88, P <0.001
P. vivax with observable Gametocytes:			
Yes	31 (68.9%)	14 (31.1%)	χ^2 with Yates' correction
No	5 (27.8%)	13 (72.2%)	= 7.27, P = 0.007
P. falciparum observable gametocytes:			
Yes	6 (46.2%)	7 (53.8%)	Fisher's exact test
No	8 (18.2%)	36 (81.8%)	P = 0.06

*body temperature >37.4 and/or reported fever, ** all cases without gametocytes had asexual stages,

*** at least some of the mosquitoes fed on each of these cases become oocyst +ve

Table 4.4 Number of malaria infected people who infected with oocysts at least some of the mosquitoes fed on them. Cases are cross-classified by (a) parasite species, (b) presence/absence of fever, (c) presence of observable gametocytes, (d) high/low parasite density. Data from village surveys and the Clinic are combined.

Fever* **/No fever** [b]	**Slides** [c]	***P. vivax*** [a] **(n = 63)**				***P. falciparum*** **(n = 57)**			
		Parasite [d] **density 40-3960/µl (n = 32)**		**Parasite density >3960/µl (n = 31)**		**Parasite density 40-3960/µl (n = 27)**		**Parasite density >3960/µl (n = 30)**	
		Oocyst		**Oocyst**		**Oocyst**		**Oocyst**	
		+ve	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**	**+ve**	**-ve**
Fever (n = 97)	Observable gametocytes +asexuals	11	6	16	7	2	1	3	4
	Only asexuals observable	1	7	2	3	3	10	4	17
	Total	12	13	18	10	5	11	7	21
No fever (n = 23)	Observable gametocytes +asexuals	1	1	3	0	1	2	0	0
	Only asexuals observable	2	3	0	0	0	8	1	1
	Total	3	4	3	0	1	10	1	1

* body temperature >37.4 and/or reported fever

Table 4.5 Comparison between each variable using Mantel Haenszel χ^2 tests

Comparison	Stratified by:	χ^2_{M-H}	*P*
P. vivax vs *P. falciparum*	Gametocytes & fever & parasite density	$\chi^2_{MH} = 1.64$	$P = 0.2$
Fever vs no fever	Species & gametocytes & parasite density	$\chi^2_{MH} = 0.01$	$P = 0.91$
Gametocytes vs no gametocytes	Fever & species & parasite density	$\chi^2_{MH} = 9.91$	$P = 0.001$
High vs low parasite density	Gametocytes & fever & species	$\chi^2_{MH} = 0.38$	$P = 0.54$

4.2.2 Comparison of the results from the village surveys and the Clinic

As shown in table 4.6 at least one mosquito became infected with oocysts in the batches fed on 10 of the 28 cases located in the village surveys (35.7%). From the Clinic, the proportion of individual patients who infected at least one mosquito was 43.4% (40/92). The difference between these two rates was not significant (χ^2 with Yates' correction = 0.26, $P = 0.6$).

Because of the possibility of heterogeneity and confounding between the results from the village surveys and the Clinic, Mantel-Haenszel χ^2 tests stratified by the village surveys/Clinic were applied to each of the comparisons made in table 4.5. The results in table 4.6 showed that only the significance of the difference between parasite species was different in table 4.6 compared with table 4.5, i.e. from the Clinic data *P. vivax* was more effective at infecting mosquitoes than *P. falciparum*, but this was not seen in the village survey data.

Table 4.6 Characteristics of 28 malaria infected cases from the village surveys and 92 cases from the Clinic and results of feeding mosquitoes on them. Results are in terms of number of cases which infected with oocysts any of the mosquitoes fed on them.

Characteristics	**Village survey**		**Clinic**		**Mantel-Haenszel tests** [a]
	Oocyst +ve (n = 10)	**Oocyst –ve (n =18)**	**Oocyst +ve (n = 40)**	**Oocyst -ve (n = 52)**	
Observable gametocytes:					
Yes	7	4	30	17	$\chi^2_{M\text{-}H} = 20.17$, $P < 0.001$
No **	3	14	10	35	
	Fisher's exact, $P = 0.02$		χ^2 with Yates' correction = 14.55, $P < 0.001$		
Parasite species:					
P. falciparum	3	10	11	33	$\chi^2_{M\text{-}H} = 11.68$, $P < 0.001$
P. vivax	7	8	29	19	
	Fisher's exact, $P = 0.25$		χ^2 with Yates' correction = 10.32, $P = 0.001$		
Fever					
Yes*	7	9	35	46	$\chi^2_{M\text{-}H} = 0.08$, $P = 0.77$
No	3	9	5	6	
	Fisher's exact, $P = 0.43$		Fisher's exact, $P = 0.10$		
Parasitaemia					
Low (40-3690/μl)	5	12	16	26	$\chi^2_{M\text{-}H} = 1.10$, $P = 0.29$
High (>3690 /μl)	5	6	24	26	
	Fisher's exact, $P = 0.44$		χ^2 with Yates' correction = 0.55, $P = 0.45$		

* Body temperature >37.4 and/or reported fever, ** All cases without gametocytes had asexual stage

[a] Stratified by village surveys/Clinic

Table 4.7 summarises the percent of infection of 120 malaria cases; 28 cases located in village surveys and 92 cases located in the Clinic on which mosquitoes were fed. 50 cases were infectious to mosquitoes, among those 10 cases were from village surveys and 40 cases were from the Clinic.

Table 4.7 Summary of proportion of malaria cases who infected with oocysts at least some of mosquitoes fed on them.

Characteristics	**Individuals who infected some mosquitoes (%)**	
	Village surveys (n = 28)	**Clinic (n = 92)**
No. positive	10/28 (35.7%)	40/92 (43.4%)
P. falciparum		
reported fever	2/7 (28.6%)	10/37 (27.0%)
no reported fever	1/6 (16.7%)	1/7 (14.3%)
P. vivax		
reported fever	5/9 (55.6%)	25/44 (56.8%)
no reported fever	2/6 (33.3%)	4/4 (100.0%)
Observable gametocytes:		
Yes	7/11 (63.6%)	30/47 (63.8%)
No	3/17 (17.6%)	10/45 (22.2%)
Fever *	7/16 (43.7%)	35/81 (43.2%)
No fever	3/12 (25%)	5/11 (45.4%)
Low parasite density (40-3,960/µl)	5/17 (29.4%)	16/42 (38.1%)
High parasite density (>3,960/µl)	5/11 (45.4%)	24/50 (48.0%)

* body temperature >37.4°C and/or reported fever

From the village surveys, among the batches of mosquitoes fed on each patient and showing positivity for oocysts, the oocyst counts per positive mosquito ranged from 6 to 470 (median 28). The geometric mean number of oocysts per infected mosquito was 3.27 for *P. falciparum* and 0.81 for *P. vivax*. The mean percentage of infected mosquitoes was higher with *P. vivax* (42.2%) than with *P. falciparum* (15.4%) This difference between the two species was statistically significant ($t = 16.92$, $df = 181$, $P < 0.001$) (Table 4.8).

From the Clinic, among the batches of mosquitoes fed on each patient and showing positivity for oocysts the mean percentage of mosquito infected was 18.3%. The geometric mean number of oocysts per infectious individual was 16.1 for *P. falciparum* and 30.9 for *P. vivax*. This difference was not significant ($t = 0.94$, $df = 38$, $P = 0.35$).

There was no significant difference between the village surveys and the Clinic data in the geometric mean number of oocysts per infected mosquito for *P. vivax* ($t = 0.88$, $df = 267$, $P = 0.37$) and also for *P. falciparum* ($t = 0.92$, $df = 44$, $P = 0.36$). However, the difference in the geometric mean number of oocysts per infected mosquito between the two species was significant for the village surveys ($t = 3.98$, $df = 55$, $P < 0.001$) and also for the Clinic ($t = 3.18$, $df = 256$, $P = 0.001$).

Table 4.8 *P. falciparum* and *P. vivax* oocysts in mosquitoes fed on cases located in the village surveys and the Clinic.

Characteristics	Village surveys		Clinic	
	Pf	Pv	Pf	Pv
Total cases				
No. of individuals	13	15	44	48
No. mosquitoes dissected	240	239	680	784
Geometric mean no. mosquitoes dissected	20.56	17.75	17.82	18.83
Range	6-36	6-35	5-34	5-40
Infectious cases				
No. infectious individuals	3	7	11	29
No. mosquitoes dissected	68	115	185	531
No. infected mosquitoes	13	44	33	225
No. oocysts	494	441	585	6,340
Geometric mean no. mosquitoes dissected	24.9	18.25	19.01	20.65
Range	12-36	10-35	6-25	5-40
Geometric mean % of infected mosquitoes	15.4	42.2	20.5	57.6
Range	10-16.7	22.8-90.9	4.3-57.9	5.7-100
Geometric mean oocyst density/infectious individual	39.9 [ab]	31.8 [ab]	16.1 [a]	30.9 [b]
Range	9-470	6-210	2-238	2-2,839
Geometric mean oocysts/infected mosquito	3.27 [a]	0.81 [b]	1.43 [a]	0.82 [b]
Range between human cases	0.5-25	0.16-7.5	0.16-40	0.04-25

Pf = *P. falciparum*, Pv = *P. vivax*

Measurements in the same row which do not differ at $p<0.05$ share a letter superscript.

4.2.3 Regression of mosquito infection on human infection

Feeds on 37 out of 58 gametocyte carriers led to oocyst production. Among those, 31 out of 37 (84%) were *P. vivax*. For subjects who infected any mosquitoes Figs 4.2, 4.3 and 4.4 show the relationships of input of parasites in the bloodmeals to output of oocysts on the mosquito's stomach. In Figs 4.2 and 4.4 the "independent" variable is natural log of gametocyte density and only the 37 detectably gametocyte positive individuals are included. In Fig 4.3 the "independent" variable is natural log of asexual parasite density and 50 patients (all positive for oocysts) are included. In Figs 4.2 and 4.3 the dependent variable is proportion of mosquitoes infected with any oocysts. As generally recommended for data in the form of proportions, the data were transformed to arcsine of square root of the proportions. In Fig 4.4 the dependent variable was the mean of log transformed oocyst load per infected mosquito. The regression analysis in Figure 4.4 is performed on the log-transformed data as this normalise the distribution of the oocyst counts. A major limitation of these regression analyses is that the "independent" variable is actually a random variable itself and therefore subject to measurement error. Accounting for measurement error may correct for the attenuation effect and provide regression estimates higher than those presented here (Carrol *et al.*, 1995) As shown in the Figures the computed regression lines all show upward slopes. However the apparent positive relationships were not statistically significant (for Fig.4.2 correlation coefficient = 0.176, t= 1.06, d.f. = 35, P = 0.29; for Fig 4.3 correlation coefficient = 0.187, t = 1.32, d.f. = 47, P = 0.19; for Fig 4.4 correlation coefficient = 0.22, t = 0.87, d.f. = 36, P = 0.39).

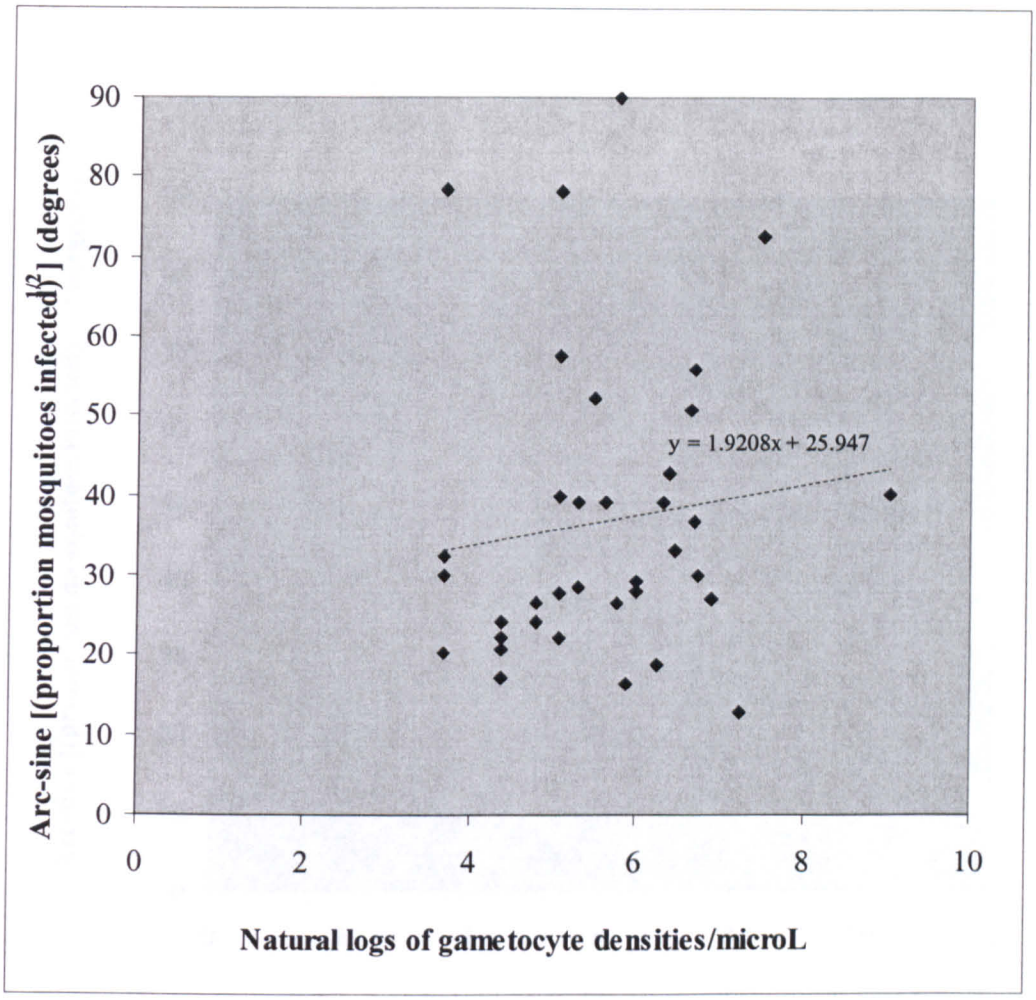

Figure 4.2 Regression of Arc-sine transformed proportion of mosquitoes infected on ln gametocyte density after feeding mosquitoes on 37 gametocyte carriers (pooled data from village survey and the Clinic and from *P. falciparum* and *P. vivax*). The parasite densities per µl were calculated from 40x density per 200 WBC (as described in chapter 2 section 2.2.1).

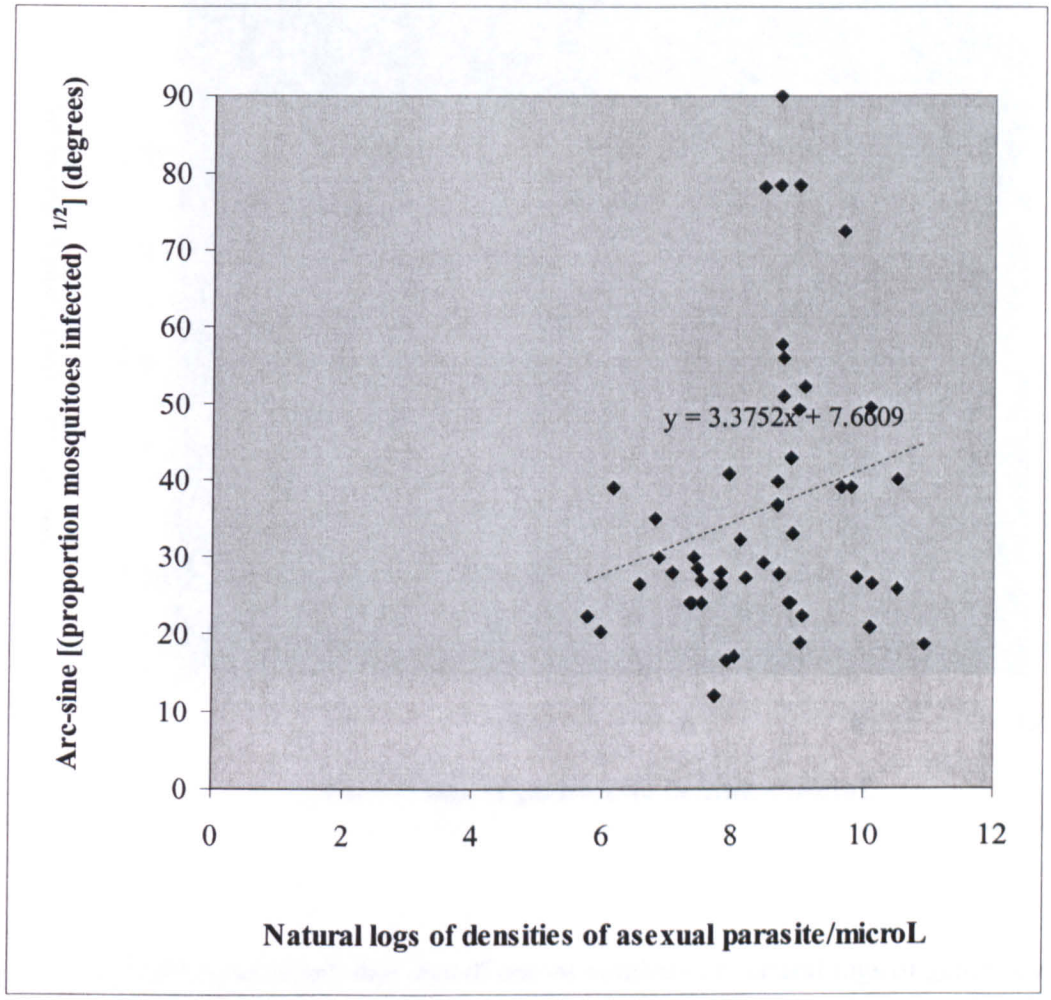

Figure 4.3 Regression of Arc-sine transformed proportion of mosquitoes infected on ln asexual parasite density from the 50 people who infected any mosquitoes (pooled data from the two parasite species and village surveys and the Clinic)

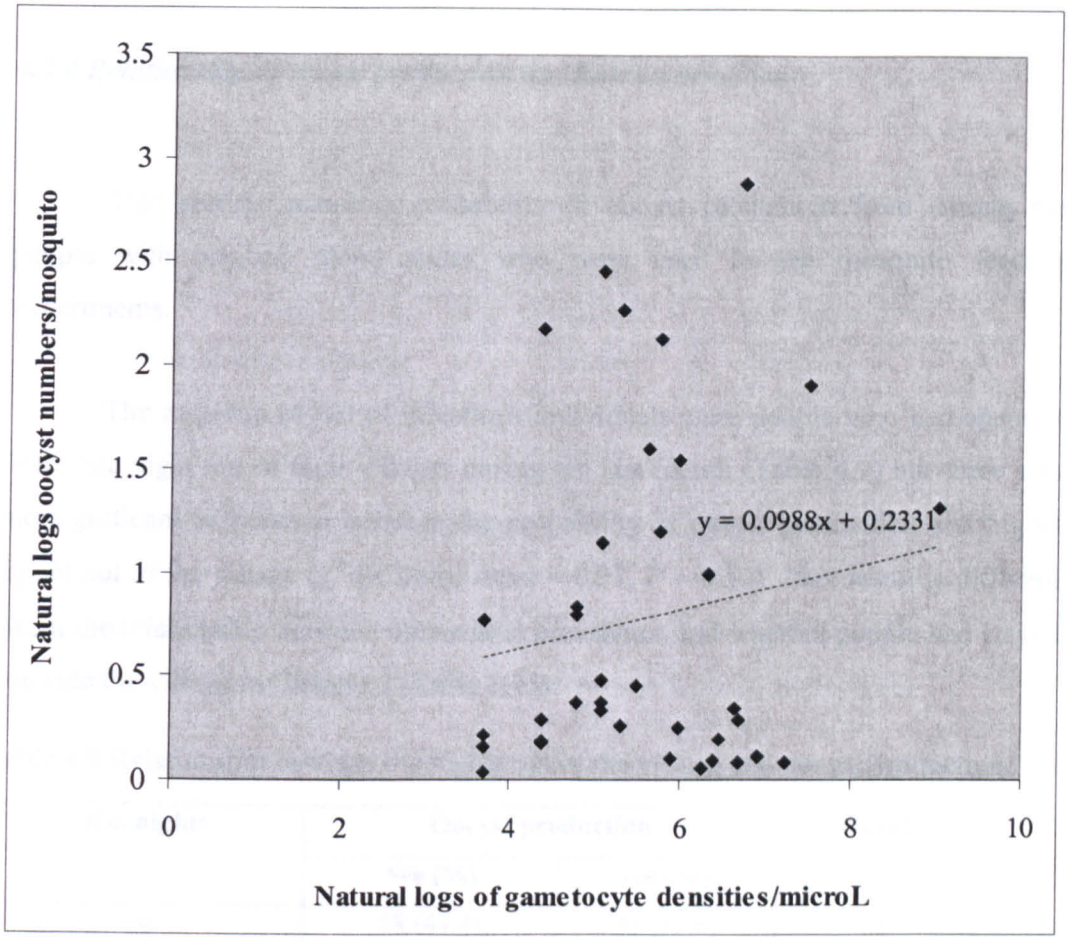

Figure 4.4 Regression of national logs of oocyst numbers on natural logs of gametocyte density in the 37 gametocyte carriers, (pooled data from village survey and the Clinic and for *P. falciparum* and *P. vivax*).

4.2.4 Relationship of oocyst production and human behaviour

This section examines probability of oocyst production from among the people with positive blood slides who were used in the mosquito feeding experiments.

The majority (44%) of infectious individuals were people who had spent at least one night out of their villages during the last month (Table 4.9) but there was no significant association between the probability of oocyst production and nights spent out of the village (χ^2 for linear trend = 0.27, P = 0.59). This result is different from the relationship between the malaria prevalence and whether people had stayed outside the village in Chapter 3 (Table 3.13).

Table 4.9 Relationship between the nights out of the village and oocyst production.

No. nights	Oocyst production		Total
	+ve (%)	-ve (%)	
0	28 (44.4)	35 (55.6)	63
1	1 (50.0)	1 (50.0)	2
2	2 (33.3)	4 (66.7)	6
3	4 (40.0)	6 (60.0)	10
4	1 (25.0)	3 (75.0)	4
5	1 (50.0)	1 (50.0)	2
6^+	13 (39.4)	20 (60.6)	33
Total	50	70	120

The proportion of people who have no nets or had nets but did not use them had about the same probability of oocyst production as those with untreated nets, who in turn had a higher rate than those with treated nets, but the trend was not significant (χ^2 for linear trend = 0.68, P = 0.41) (Table 4.10). This finding was different from table 3.14 when the comparison was done between the slide positivity

and those five groups, and a highly significant trend was found (χ^2 for linear trend = 22.98, P <0.001).

Table 4.10 Mosquito net status and probability of oocyst production.

Net status	Oocyst production		Total
	+ve (%)	-ve (%)	
Treated nets	8 (36.4)	14 (63.6)	22
Untreated nets in treated villages	22 (40.0)	33 (60.0)	55
Untreated nets in untreated villages	12 (46.2)	14 (53.8)	26
Nets owned but not used or no net	8 (47.1)	9 (52.9)	17
Total	50	70	120

4.3 Results from PCR study

4.3.1 Test of people with negative slides who were fed on by mosquitoes

Blood from 58 subjects with negative blood slides who were fed on by mosquitoes were tested for *P. falciparum* infections using nested PCR (Pf CRT). This technique has demonstrated a high prevalence of sub-microscopic asymptomatic *P. falciparum* infections among inhabitants of malaria-endemic countries (Bottius *et al.*, 1996; Contamin *et al.*, 1996; Farnert *et al.*, 1997; Babiker *et al.*, 1999). Some subjects with negative slides from the present study may have infection undetectable by microscopic examination so this technique would allow us to answer the question whether some very low density infections are infectious to mosquitoes and are therefore part of the reservoir of infection which keeps transmission going.

4.3.1.1 Methods

When thick blood smears were collected for microscopical examination, one drop of blood was spotted and allowed to dry completely on glass fibre filter paper. Then *An. minimus* A were allowed to feed on the subjects' arms for 15-20 minutes. The dried blood spots were placed in individual self-seal polythene bags and stored at room temperature before being used for PCR analysis.

A nested PCR method was used to amplify a portion of the Pf CRT gene (164bp) (Djimde *et al.*, 2001). The first round of PCR products was generated using a small piece of glass fibre membrane (0.3x0.3 mm). Nest 1 PCR reaction mixtures contained 1mM of forward and reverse primers, 200mM dNTPs, 1.5mM $MgCl_2$ and 2.5U-1 Taq DNA polymerase (BIOTAQ™, Bioline, London, UK) with the buffer supplied by the manufacturer. Nest 1 PCR conditions were as described by (Djimde *et al.*, 2001). The nest 2 PCR reaction was performed with primers TCRD2 and TCRD3 (5'-AGGTTCTTGTCTTGGTAAATTTGC-3'), one micolitre of each nest 1 PCR product was used. As positive controls the 3D7 and HB2 laboratory clones of *P. falciparum* were used. Amplification conditions were 95°C for 3 min initial denaturation, 94°C for 30 sec, 56°C for 20 sec and 65°C for 30 sec, for 30 cycles and a final extension at 65°C for 5 min. PCR amplifications were performed in a Peltier Research Cycler (PTC-200, MJ Research Inc. USA). PCR products were resolved by electrophoresis through 4% MetaphoreTM agarose gels (FMC BioProducts, Rockland, Maine, USA) in 0.5X TBE buffer, stained with ethidium bromide and visualised by ultraviolet transillumination.

4.3.1.2 Results

58 subjects with negative slides were fed on by mosquitoes. Among those, 28 subjects were from the village and 30 were from the Clinic. All of them gave negative results (i.e. produced no oocysts) on mosquito feeding. However, the results from the PCR technique showed that 30% (17 out of 58) were positive for *P. falciparum* (Table 4.11). Figure 4.5 shows an example of a gel with positivity for *P. falciparum* in lane 5, 8, 13,14 and 15. There was no association between PCR positivity for *P. falciparum* and fever (χ^2 with Yates' correction = 0.01, $P = 0.9$). However, the difference in probability of oocyst production between those slide positive by microscopy (14 patients out of 57 producing oocysts) and those positive by PCR (0/17) was significant (Fisher's exact test, $P = 0.03$) (Table 4.12). The slide negative subjects could not be tested by PCR for *P. vivax*. For subjects, scored as gametocyte negative by microscopy and who were able to infect mosquitoes, it was hoped to use the RT-PCR method which could distinguish gametocytes from asexual stages. In this way it was hoped to determine whether a small number of

gametocytes did actually exist at a density too low to detect by microscopy. However, Dr. Georges Snounou advised that the RNA required for this method would not have survived transport without deep refrigeration and application of this method were therefore not attempted.

Table 4.11 Subjects with negative slides from the village and the Clinic who were fed on by mosquitoes and tested for *P. falciparum* using nested PCR technique.

Village surveys/Clinic	**Fever*/ no fever**	**Oocyst production**		**PCR of blood smear tested for *P. falciparum***	
		+ve	**-ve**	**+ve**	**-ve**
Village surveys (n = 28)	Fever (7)	0	7	3	4
	No fever (21)	0	21	4	17
Clinic (n = 30)	Fever (12)	0	12	2	10
	No fever (18)	0	18	8	10
Total		0	58	17	41

* body temperature >37.4°C and/or reported fever

Table 4.12 Oocyst productivity of *P. falciparum* from microscopy and PCR results

P. falciparum	**Oocyst +ve**	**Oocyst -ve**	**Statistical test**
Slide +ve (From table 4.3)	14	43	Fisher's exact test, $P = 0.03$
Slide –ve but PCR +ve	0	17	

Figure 4. 5 A representative example of the results from negative blood smears using the nested PCR assay for *P. falciparum*. Lanes 1 and 2 are the positive controls (3D7 and HB2, respectively). Lane 16 is the ladder marker. Lanes 3-15 correspond to 13 of the slide negative subjects (by microscopy), with lanes 5,8,13, 14 and 15 showing positivity for *P. falciparum* (by PCR).

4.3.2 PCR determination of malaria parasite species in human and vectors fed on them

Some malaria infections may contain more than one species but only a dominant species may be observed by microscopy. The PCR technique would allow the identification of the parasite species in the mosquito's midgut and compare them with the species identified by microscopy on finger pricks from the person on whom the mosquito fed.

4.3.2.1 Methods

Mosquitoes were dissected 7-9 days after feeding. Dissections were made using minuten pins that were sterilised by flaming every time an infected midgut was handled to minimise the risk of DNA contamination. Midguts were examined for the presence of oocysts under a compound microscope. Then positive or negative

midguts were transferred to separate labelled eppendorf tubes containing two drops of normal saline. Specimens were then stored at –20°C.

DNA extraction from mosquito samples was carried out as described by Ranford-Cartwright *et al.* (1991). *Plasmodium* species were determined using nested PCR as described elsewhere (Snounou *et al.*, 1993a; Singh *et al.*, 1999). Briefly, the PCR buffer and oligonucleotide primers were removed from the freezer. The master mix was prepared from PCR buffer and, oligonucleotide primers. The final $MgCl_2$ concentration was 2 mM. Each oligonucleotide primer was used at a final concentration of 250 nM. dNTPs were added to give a final concentration of 125 μM and then appropriate amounts of Taq polymerase were added. The contents were mixed and then 20 μl of the master mix was aliquotted in to each tube. DNA template (usually 1 μl) was added. The positive controls were 1μl of genomic DNA corresponding to approximately 50 nuclei of *P. falciparum* (purified from the *in vitro* cultured 3D7 line) or *P. vivax* obtained from a blood sample from an infected chimpanzee provided by William E. Collins from CDC Atlanta, Georgia USA. When aliquoting the product of the Nest 1 reaction as a DNA template for the Nest 2 reaction, the DNA template aliquot was removed from the Nest 1 tube and then added to the Nest 2 tube. Cycling conditions for the PCR amplifications were 25 cycles: 95°C, 5 min; 58°C, 2 min; 72°C, 2min; 94°C, 1 min; 58°C, 2 min; 72°C, 5 min final extension. These conditions have been provided using a PTC-100 Thermal cycler (MJ Research Inc., USA). After amplification the PCR products were run on agarose gels and stained with ethidium bromide. The PCRs were analysed regardless of the number of oocysts determined by microscopy.

4.3.2.2 Results

The results are shown in table 4.13 and Figure 4.6. None of the midguts were found by PCR to be infected with *P. falciparum*. However, midgut infection with *P. vivax* were found in mosquitoes fed on 23 human donors. Thirteen of these were in mosquitoes with microscopically visible oocysts. Eight were in mosquitoes that did

not themselves carry visible oocysts but they came from donors which had been shown to be infectious to mosquitoes by the fact that other mosquitoes fed on the same donors had visible oocysts. Finally in two cases PCR positivity was found where none of the mosquitoes fed on these two donors had visible oocysts. Those 23 cases of PCR positivity for *P. vivax* all came from donors with visible *P. vivax* infections. None of the donors with visible *P. falciparum* infections also carried cryptic *P. vivax* infections which could yield *P. vivax* PCR positivity in mosquitoes fed on them.

Table 4.13 Results of PCR for *Plasmodium* species in mosquito midguts, classified by species infecting the donor's blood, and whether oocysts were visible by microscopy in any of the mosquitoes fed on the same donor.

Species infecting the blood of donor	**Oocysts visible on any of the mosquitoes fed on the same donor**	**Mosquitoes with visible oocysts**				**Mosquitoes with no visible oocysts**			
		P. falciparum		***P. vivax***		***P. falciparum***		***P. vivax***	
		PCR +ve	**PCR -ve**	**PCR +ve**	**PCR -ve**	**PCR +ve**	**PCR -ve**	**PCR +ve**	**PCR -ve**
P. falciparum	Yes	0	12	0	0	0	10	0	0
	No	-	-	-	-	0	27	0	0
P. vivax	Yes	0	0	13	13	0	0	8	17
	No	-	-	-	-	0	0	2	7

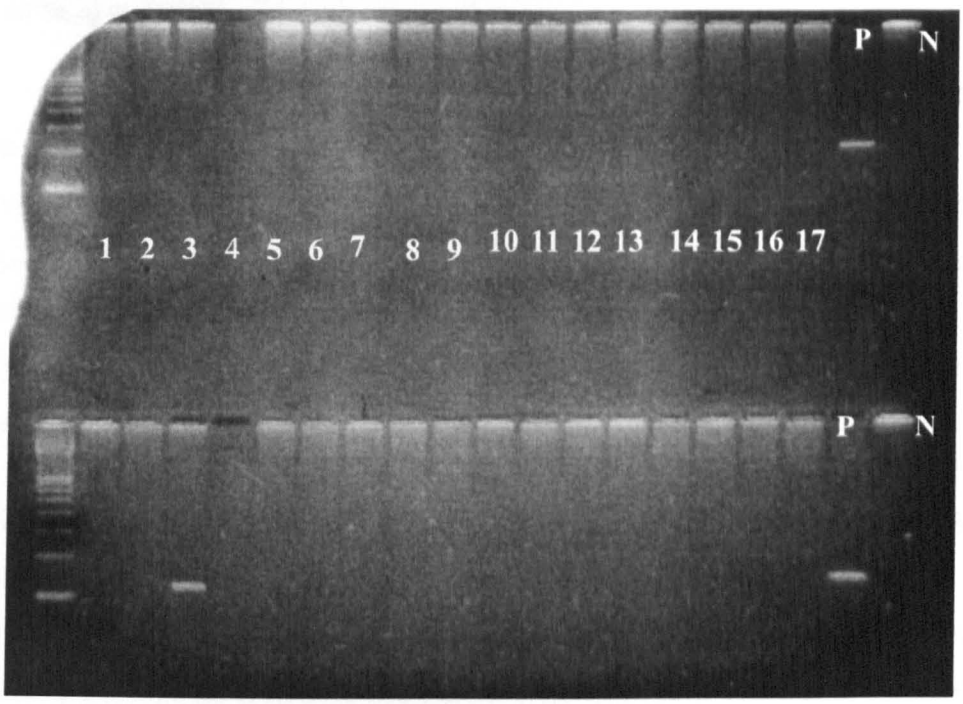

Figure 4. 6 A representative example of the results of PCR of oocysts from mosquito midguts. Upper row is for the test for *P. falciparum* and lower row is the test for *P. vivax*. Lanes 1-6 are the samples from negative midguts by microscopy and lanes 7-17 are the positive samples by microscopy. The positive control is represented with letter "P", and "N" is a negative control. On this gel only lane 3 was positive and it was positive for *P. vivax*.

4.4 Comparison of gametocyte densities in blood meals with blood from finger prick

It has been shown that some cases with undetectable gametocytes could infect mosquitoes. There is approximately 2 mg (= 2 μl) of blood in an *An. arabiensis* blood meal (Vaughan *et al.*, 1991) but only about 0.12 μl (detailed in section 4.4.1) from a blood smear is observed on a microscope slide. Thus when a small number of oocysts is produced in a mosquito fed on blood in which no gametocytes were observed this could be explained by the chance absence of gametocytes from the small volume observed in the blood smear. However, the maximum number of oocysts in a mosquito fed on blood without observed gametocytes was 300 (Table 4.14). This was from subject MC48 who yielded 13/24 mosquitoes with more than 100 oocysts and a geometric mean of 104.8 oocysts per mosquito There must have been at least as many female gametocytes, plus enough male gametocytes to fertilise them, in the blood meal. Even allowing for the smaller volume of the blood smear than the blood meal it is difficult to understand how no gametocytes were observed in cases MC17, MC18 and MC48. This suggests the question "Do gametocytes selectively enter the mosquito's proboscis during blood feeding?"

In order to investigate this question the density of gametocytes was determined in finger prick blood smears and on smears made from blood meals immediately after mosquitoes had fed on the same human subjects.

Table 4.14 Distribution of oocyst number from the infectious individuals without observable gametocytes.

Human subject no.	Mosquito no.	No. oocysts	No. oocyst negative mosquitoes	Geometric mean number oocysts among positive	Williams' means	Minimum no. of female gametocytes per 0.12 µl of blood
37	1	5	18	7.07	0.52	0.03
	2	10				
78	1	2	8	1.91	0.61	0.04
	2	6				
	3	2				
	4	2				
	5	1				
	6	1				
84	1	1	12	1.12	0.23	0.01
	2	1				
	3	1				
	4	2				
	5	1				
	6	1				
MC13	1	4	7	5.65	0.53	0.03
	2	8				
MC14	1	2	9	2.83	0.23	0.01
	2	4				
MC17	1	200	20	12.10	0.70	0.04
	2	4				
	3	4				
	4	3				
	5	27				
MC18	1	11	17	29.62	0.93	0.06
	2	100				
	3	100				
	4	7				

MC19	1	41	17	12.81	0.32	0.02
	2	4				
MC21	1	2	22	2.0	0.05	0.003
MC38	1	5	15	2.34	0.30	0.02
	2	3				
	3	2				
	4	1				
MC48	1	109	1	104.8	86.9	5.2
	2	67				
	3	40				
	4	100				
	5	250				
	6	300				
	7	58				
	8	115				
	9	200				
	10	200				
	11	100				
	12	100				
	13	80				
	14	70				
	15	90				
	16	110				
	17	80				
	18	150				
	19	90				
	20	70				
	21	70				
	22	100				
	23	200				
	24	90				
MC75	1	3	5	3.0	0.60	0.04
MC95	1	2	5	2.0	0.48	0.03

4.4.1 Methods

Mosquitoes were fed on 6 volunteers (designated by the numbers 1, 2, 3, 4 for those who had observed gametocytaemia and 5 and 6 for patients who had no observable gametocytes and only asexual stages). Cups containing 15 *An. minimus* species A were placed in contact with the skin of the volunteers for 15-20 minutes. Immediately after completion of feeding, 5 engorged mosquitoes per human subject were killed and the midguts were removed under a binocular microscope. The midgut contents were smeared on to microscope slides. These slides were dried for one day at room temperature, stained with Giemsa solution for 20 min, washed with water, and dried. The count of gametocytes was done using a 100x oil immersion objective. Thick blood smears from finger pricks on the same patients were taken at the time of the blood feeding and stained and examined in exactly the same way. On five random sections of each human blood smear gametocytes were counted per 200 WBC, thus the gametocytes associated with a total of 1,000 WBC was observed. Assuming that there are 8,000 WBC per microlitre (Wilcox, 1960), 0.12 µl of blood from the finger prick was counted.

4.3.2 Results

In the thick blood films from finger pricks volunteers 1, 2, 3 and 4 harboured mean gametocytaemias of 93, 10, 206 and 63/1,000 WBC, respectively (Table 4.15). One case had a *P. vivax* infection and the other three *P. falciparum*. No gametocytes were observed for case nos. 5 and 6. The geometric mean counts of gametocytes in smears from mosquito midguts are shown in table 4.15. In two cases, all five of mosquitoes ingested gametocytes but in the other two only 3 out of 5 of the mosquitoes ingested any observable gametocytes.

In Table 4.15 we did not convert into microL (μl) because there may be concentration of the blood after consumption by the mosquitoes so the density of 8,000 WBC/μl may not apply in the blood meal. However, we believe that this concentration would equally affect the gametocytes and WBC. Thus to assess the finger prick: blood meal ratios of gametocyte concentration gametocyte densities per 1,000 WBC was used.

In this study, the ratio between the densities of ingested gametocytes and the gametocytaemia observed from finger pricks were 1.3, 1.5, 4.2 and 5.7 in volunteers 1, 2, 3 and 4, respectively. The gametocyte density appeared to be lower in the mosquito blood meals than in the finger pricks, but the difference was not significant ($t = 1.1$, $df = 3$, $P = 0.3$). It is concluded that there was no evidence from this experiment to support the hypothesis that gametocytes are able to have themselves selectively taken up in the mosquito's blood meal.

Table 4.15 Gametocyte densities counted from finger prick and the blood meals of *An. minimus* species A.

	Case 1 (Pf)	Case 2 (Pf)	Case 3 (Pv)	Case 4 (Pf)	Case 5 (Pf)	Case 6 (Pf)
No. blood meals with gametocytes/no. dissected:	3/5	5/5	5/5	3/5	0/5	0/5
Range of no. of gametocytes/ 1,000 WBC in smears from blood meals:	0-90	5-12.5	15-125	0-2.5	0	0
Geometric mean gametocyte density from blood meal per 1,000 WBC	71	6.5	48.5	11	0	0
SD	42.7	2.7	42.6	10.3	0	0
95% CI:	32.5-154	4-10.5	18-131	1.5-80	0	0
Geometric mean of gametocytes from finger prick per 1,000 WBC	93	10	206	63	0	0
SD	27.1	5.4	38.4	34.9	0	0
95% CI:	56.5-153	5.5-18	165.5-256.5	50.5-78.5	0	0
Ratio finger prick: blood meal:	1.3:1	1.5:1	4.2:1	5.7:1	0	0

Pf = *P. falciparum*, Pv = *P. vivax*

4.5 Discussion

The estimate of the infectiousness of the *Plasmodium* reservoir to mosquitoes is of interest in understanding the epidemiology of malaria and its changes after application of certain types of control measure. This infectiousness can be estimated in several ways. Feeding mosquitoes on members of the human population is a direct approach. This chapter describes the infectiousness of individuals to mosquitoes and attempts to identify the main reservoir of malaria infection either from people who feel ill enough to go to the malaria Clinic, or people who have remained in their villages.

4.5.1 Differences in percent of infection

The results from the experiments on mosquitoes showed the infectiousness of aymptomatic carriers as well as symptomatic ones (Table 4.3). Sattabongkot *et al.* (1991) reported that symptomatic infections in adults could infect mosquitoes. However, Tchuinkam *et al.* (1993) showed that 62% of gametocyte carriers from Cameroon were infective. Boudin *et al.* (1993) found that all age groups from Burkina Faso were infectious to mosquitoes, while Muirhead-Thomson (1957) and Graves *et al.* (1988) found that low percentages of the individuals studied were infectious to mosquitoes.

The differences in percentages mentioned above might be explained by several possible factors. First, method of feeding: a recent study comparing the infectivity of gametocyte carriers to mosquitoes, using membrane and direct feeding, found significantly higher numbers of mosquitoes infected and higher oocyst burdens in mosquitoes fed direct on the skin (Bonnet *et al.*, 2000). Sattabongkot (2002) showed that direct feeding was more effective than membrane feeding for all parameters when *An. dirus* were fed on *P. vivax* patients. Conversely, Vanderberg (1980) reported that infectivity in mosquitoes fed through a membrane usually equalled or exceeded infections by direct methods. However, most studies gave better results in direct feeding than membrane feeding. Thus the studies using direct

feeding may provide a more reliable estimate of the infectious reservoir. Second, recruited subjects: in several studies mosquitoes were fed on individuals selected randomly and not on the basis of gametocytaemia (Muirhead-Thomson, 1957; Githeko *et al.*, 1992; Boudin *et al.*, 1993). But in other studies mosquitoes were fed on selected gametocyte carriers (Sattabongkot *et al.*, 1991; Touré *et al.*, 1998) or parasitaemic cases with or without gametocytes (as in the present study).

Another relevant factor is variability in mosquito populations. Several studies showed that variability within mosquito populations may affect the pattern of transmission (Dye and Hasibeder, 1986; Koella and Lyimo, 1996). Kitthawee *et al.* (1992) showed that the largest size category of *An. dirus* developed the highest mean number of oocysts. Two years later, the same result was reported in *An. gambiae* (Lyimo and Koella, 1992). Kelly and Edman (1992) also showed that large *Aedes aegypti* infected with *P. gallinaceum* was more infective than small mosquitoes. A similar relationship may exist within and between different species of *Anopheles*. The number of blood meals may also affect the infection rate. Hurd *et al.* (1995) suggested that multiple blood-feeding behaviour might play an important role in the infection of mosquitoes, especially in areas of low malaria transmission. Ponnudurai *et al.* (1989b) showed that post-infection blood meals increased the sporozoite production. Thus it would be useful to compare the infectivity between the main vectors in the same transmission areas. A careful comparison of *An. dirus*, *An. minimus* and *An. maculatus* of infectivity in relation to size and blood-feeding behaviour would be of interest.

Another factor may arise from genetic change in mosquito colonies continuously maintained for a long time. Despite inbreeding, variability in susceptibility of members of mosquito colonies is possible (Rutledge *et al.*, 1969; Sattabongkot *et al.*, 1991). Variability in oocyst loads occurs even in populations that have undergone many generations of selection for susceptibility to the parasite (Feldmann and Ponnudurai, 1989). Laboratory-adapted insects are normally used and these could differ from wild mosquito populations in their susceptibility to infection. In every population of mosquitoes, whatever their susceptibility for a

particular species of *Plasmodium*, there are individuals with a variety of rates of digestion and formation of the peritrophic membranes, resulting in a wide range of oocyst numbers (Ponnudurai *et al.*, 1989a).

4.5.2 Low densities or undetectable gametocytes

Absence of transmission-blocking antibodies (Mendis *et al.*, 1987; Ponnudurai *et al.*, 1989; Peiris *et al.*, 1988), gametocyte maturity (Hawking *et al.*, 1971), good-quality gametocytes (Pampana, 1965; Nedelman, 1990), and an optimum number of these stages, are needed for high infection rates and there is also a need for the best possible way of bringing parasites and mosquitoes together (Ponnudurai *et al.*, 1989). The results in the present study showed that cases with low gametocyte densities or a confirmed lack of observable gametocytes were infectious and, conversely, cases were found with high densities of gametocytes, which were not infectious. This paradox of infectivity has been reported in most studies assessing human malarial infectivity to mosquitoes (Jeffery and Eyles, 1955; Rutledge *et al.*, 1969; Gamage Mendis *et al.*, 1991; Sattabongkot *et al.*, 1991; Tchuinkam *et al.*, 1993). Sattabongkot *et al.* (1991) found that one third of gametocyte carriers failed to infect mosquitoes and cases that infected the highest proportion of mosquitoes also produced the highest mean oocyst levels. However, Ponnudurai *et al.* (1989) found an increase in the number of oocysts when the gametocytes were reduced by 3-fold or more. Bonnet *et al.* (2000) showed that 14.3% of individuals without visible gametocytes were able to infect mosquitoes by direct or membrane feeding. A weak relationship between gametocyte density and infectiouness of individuals to mosquitoes was observed. Huber *et al.* (1998) found cases of PCR positivity for *Plasmodium* but negativity by microscopy. Some of the mosquitoes fed on these cases became oocyst positive. It can be suggested that there were "hidden" but transmittable gametocytes in the blood which can move in and out of the peripheral blood, and were not detectable at the time of finger prick sampling. It has been suggested that the prevalence of gametocyte carriers is not a good indication for the infectiousness of a population to mosquitoes (Jeffery and Eyles, 1955; Muirhead-Thomson, 1957; Carter, and Gwadz, 1980; Carter and Graves,

1988; Boudin *et al.*, 1993). These factors may be able to explain the lack of correlation between gametocyte densities and infectiousness in the present study (Figure 4.2 & 4.4).

It would be helpful to be able to detect gametocytes at a density below the threshold of microscopic detection. Reverse transcriptase polymerase chain reaction (RT-PCR) allows for detection of gametocytes of malaria parasites. A method, which combines RT-PCR and nested PCR, can readily detect 1-2 gametocytes per µl blood with total specificity with respect to asexual stages (Babiker *et al.*, 1999). Thus, this method should be suitable for detection of carriers of low numbers of gametocytes, who may play a major role in malaria transmission. However, this method has a limitation that at present it can detect only *P. falciparum.* It would be valuable if methodology could be developed to detect *P. vivax* gametocytes by RT-PCR. Unfortunately, in the present study it was not possible to detect *P. falciparum* gametocytes by RT-PCR technique, because the samples were not preserved adequately to retain RNA. However, the blood spots collected on glass fibre filter papers from 58 subjects with no microscopically detectable parasites were tested by PCR for *P. falciparum* and the subjects were fed on by mosquitoes. The results showed that 30% of negatives by microscopy were positive by PCR but none of them yielded oocysts (Table 4.11). However, these asymptomatic carriers with very low density parasitaemia may play an important role in maintaining transmission because their level of parasitaemia may fluctuate from day to day and sometimes reach mosquito-infective densities. Any attempts at using mass drug treatment to eliminate the reservoirs of transmission should consider PCR diagnosis to detect low density parasitaemia which could give rise to a mosquito transmission density on a later occasion.

4.5.3 Asexual stages and infection

No significant association was found between asexual parasite densities and oocyst production (Figure 4.3). Boudin *et al.* (1993) also showed that high and low infective groups had the same mean levels of mean parasite loads. Thus he could not explain the level of infectiousness by the level of asexual parasites. However, the results from animal models such as the studies in avian (Eyles, 1952), rodent and simian malaria (Carter, and Gwadz, 1980) showed a strong indication that the period of high asexual parasitaemia is associated with reduced infectivity of the gametocytes to mosquitoes.

4.5.4 Gametocytes from humans to mosquitoes

Unless a major error has been made in the understanding of the life cycle of *Plasmodium* for the last 100 years, it must be assumed that gametocytes were present but could not be observed in all cases where oocysts were produced. The data on gametocytes in blood meals showed that the density was not significantly higher (and appeared to be lower) in the blood taken up by mosquitoes than in the blood from finger pricks, indicating that the gametocytes do not selectively enter a mosquito's proboscis. The occurrence (Table 4.14) in some cases of high oocyst counts in mosquitoes fed on blood with no observable gametocytes remains unexplained. The results from the present study showed that the maximum number of oocysts in a mosquito fed on blood without observed gametocytes was 300 (Table 4.14). That is at least 300 female gametocytes must have existed in the 2 μl of mosquito blood meal. Thus one would have expected at least 18 female gametocytes (plus male gametocytes) in the 0.12 μl of blood which were examined on the corresponding slide but, despite repeated examination, none were seen.

4.5.5 The reservoir of infection

From the results, an attempt was made to estimate the number of people in the catchment area of the Clinic who were reservoirs of infection. On the basis of the number of patients visiting the Clinic per day and the catchment population from which the patients come (Figure 4.7), it was concluded that the main reservoir of infection for mosquitoes was not in patients feeling ill enough to be motivated to come to the Clinic. Among villagers occurrence of fever is a strong indicator of likely malaria infection (Table 3.16). Thus fever is an indicator of likelihood of being part of the infectious reservoir for mosquitoes. However as shown in tables 4.3, 4.5 and 4.6 among those who have microscopically visible malaria infection, fever was not associated with greater likelihood of being infectious to mosquitoes. Table 4.7 indicates that more villagers are infectious reservoirs for *P. vivax* than for *P. falciparum*. The calculations in Figure 4.5 do not take into account the fact that the infected people found in the villages will remain infected for several days, whereas a new group of about 9 people goes to the Clinic every day. It would be useful to know for how many days people remain infectious to mosquitoes, but ethically one cannot test this. The data in Figure 4.5 from the present study suggest that directing an anti-gametocyte drug only to the clinic patients would be ineffective, but directing the drug to all feverish patients in the villages could have a major impact on the reservoir of infection. In view of the current interest in anti-gametocyte drugs (e.g. Chavalitshewinkoon-Petmitr *et al.*, 2000; van Vugt *et al.*, 2000; Targett *et al.*, 2001), these data may be of use in deciding how such drugs would have to be targeted to have an impact on transmission.

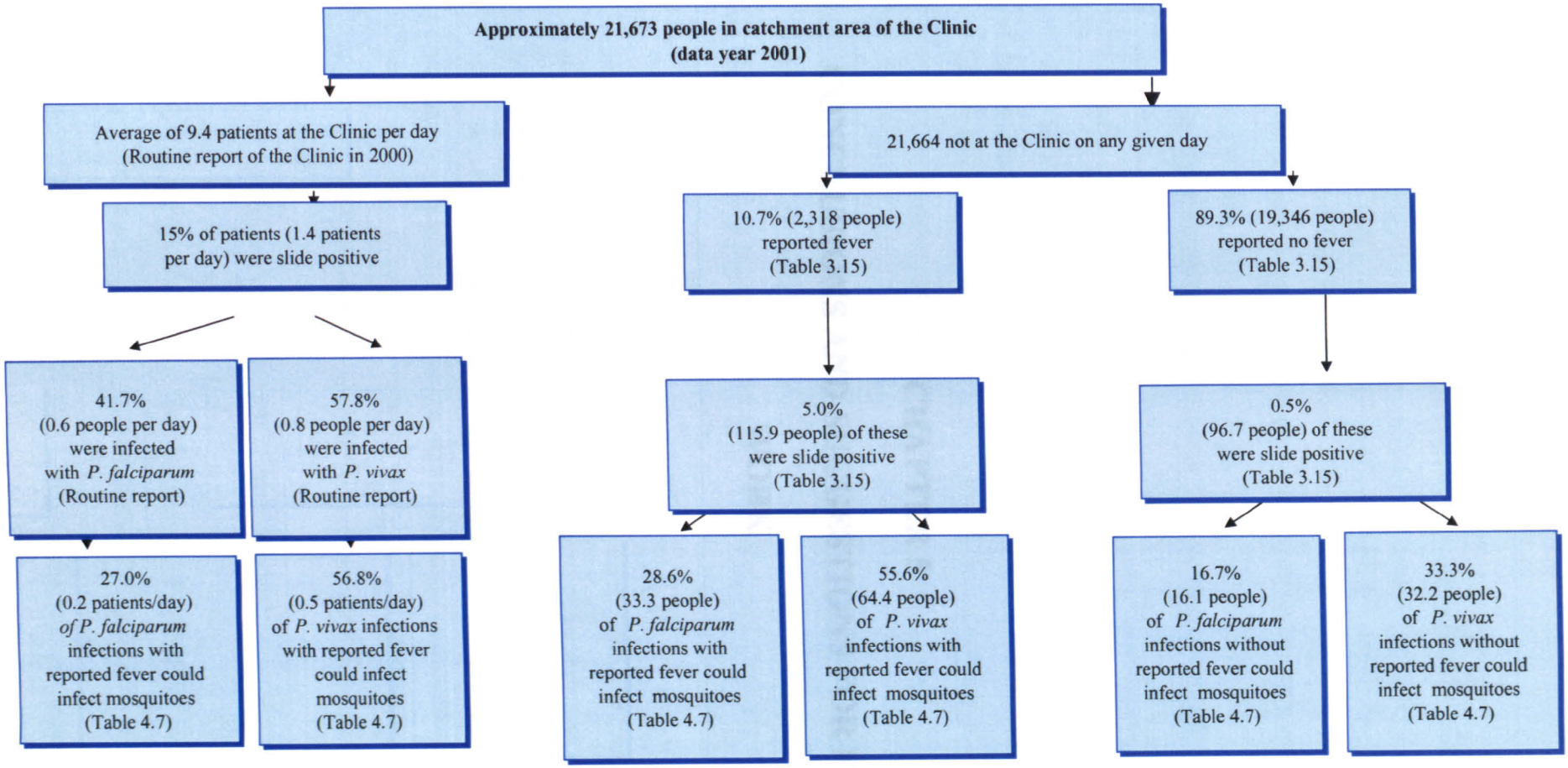

Figure 4.7 Diagram to show location of the reservoirs of infection.

CHAPTER 5

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

5.1 Conclusions from Chapter 3

5.1.1 Mass blood surveys were carried out primarily to determine the prevalence of asymptomatic malaria in the general population of various age groups living in an unstable transmission area, and also to find malaria infected individuals for the mosquito infection studies (chapter 4). The surveys were carried out in 30 villages of three cantons and about 67% of people who provided blood samples were interviewed using a structured questionnaire. The parasite rate was 0.7% (57/7,812) with 24 *P. falciparum*, 32 *P. vivax* and 1 mixed infection being found (Table 3.4). This slight excess of *P. vivax* was seen in the two surveys at different seasons. A significantly higher prevalence was recorded for men than for women (Table 3.6). Observable gametocytaemia for *P. vivax* and *P. falciparum* was seen in about 46.9% and 29.2% of cases of parasitaemia for the two species.

5.1.2 The results from the interviews of recall of any malaria episode revealed that the highest percentage of reports was for the month of May (Figure 3.4). This finding is similar to the reported incidence of clinically diagnosed malaria for Thailand. When people wished to have a blood test and obtain treatment when they thought they had malaria, the Clinic and hospitals were most commonly used. Self-medication was rare. Thus, treatment-seeking behaviour for malaria infection in Thailand is entirely different from Africa, India or some countries in south east Asia such as the Cambodia or Vietnam where most people go to shops or the private sector for anti-malarial drugs. The widespread improper use of anti-malarial drugs is thought to have been the cause of a major increase in malaria mortality in Africa due to the rise of drug resistance. Thus the Thai system of limiting anti-malarial drugs to a well organised public sector is certainly to be recommended.

5.1.3 Approximately 20% of the surveyed population had stayed outside their village for one or more night during the previous month and this group contributed 52.8% of total slide positivity. The main purpose of staying outside their villages was for looking after crops and the farmers mostly live in the temporary huts or tents

which expose them to mosquitoes. These people presumably become an important reservoir of infection when they return home.

5.1.4 The results from the present study showed that more than 95% of people in this area owned mosquito nets. Most of them reported using nets every night but a few never used them. However, it was reported that less than 50% of the nets had been treated with insecticide. A significantly higher malaria prevalence was found in the people who reported not having any form of mosquito protection or who had nets but did not use them or who had untreated nets, when compared with those who used treated nets (Table 3.14).

5.1.5 All those with positive slides who had a body temperature >37.4°C reported fever. Contrary to expectation *P. falciparum* infections did not cause more fever than did *P. vivax* infections. The prevalence of asymptomatic malaria was 47.1% among those who were slide positive which corresponded to about 0.5% of the interviewed population. Asymptomatic cases were observed more frequently in adults than children and in males than females, but the differences were not significant (Table 3.17).

5.1.6 From the Clinic, 49 people with *P. falciparum* infections and 52 with *P. vivax* infections were interviewed (Table 3.18). Most people who came to the Clinic reported fever in the previous week and had a body temperature >37.5°C. More than half of the infections detected at the Clinic had a high parasitaemia (>3,960 parasites/μl).

5.2 Conclusions from Chapter 4

5.2.1 People infected with either *P. vivax* or *P. falciparum* detected via the village surveys or the Clinic could infect mosquitoes. The geometric mean number of oocysts per infected mosquito differed significantly between *P. falciparum* and *P. vivax* for the village surveys and also for the Clinic (Table 4.8). Approximately, 50% of people with observable gametocytes failed to infect mosquitoes in the cases of *P.*

falciparum and 32% for *P. vivax*, but the difference was not significant. As in other studies, there were some individuals without observable gametocytes who could infect mosquitoes. However, there was a significant association of probability of mosquito infection with presence of observable gametocytes and it was concluded that *P. vivax* infections were significantly more likely to infect mosquitoes than were *P. falciparum* (Table 4.7). There was no significant difference in mean number of oocysts per infected mosquito for *P. vivax* or *P. falciparum* between the village surveys and the Clinic (Table 4.8).

5.2.2 None of 58 subjects with slides with no observable infection who were fed on by mosquitoes yielded any oocysts (Table 4.12). PCR showed that about 30% (17/58) of cases with negative slides were positive for *P. falciparum* infection (Table 4.11). Tests for parasite species in mosquito midguts using nested PCR showed that none of the midguts were positive for *P. falciparum* by PCR. This applied to individuals who could or could not infect mosquitoes with oocysts. For *P. vivax*, PCR positive midguts were found, including in cases where there were no visible oocysts (Table 4.13).

5.2.3 The gametocyte density was no higher in the mosquito blood meals than in the finger pricks. Thus there was no evidence for selective uptake of gametocytes by mosquitoes and cases with no observable gametocytes but able to yield numerous oocysts in mosquitoes remain unexplained (Table 4.15).

5.2.4 People located in the village surveys who had reported fever and body temperature >37.4°C were much more likely to be parasite positive than people without parasite symptoms. Symptomatic or non-symptomatic people with parasites were equally able to infect mosquitoes (Table 4.7). An estimate of where the main reservoir of infection was to be found was calculated from (a) the number of people attending the Clinic daily and the catchment population of the Clinic (b) the prevalence of infection in people at the Clinic and in the villages; (c) the probability of oocyst production from these categories of people. It was concluded that the main reservoir of infection for mosquitoes was in people who not ill enough to go to the

Clinic but who had some feverish symptoms (Figure 4.7). These should be the main target of a campaign of mass drug administration with anti-gametocyte drugs.

5.3 Limitations of the study

5.3.1 No mosquito feeding was done on children less than 15 years of age as the Thai ethical committee did not allow this. Thus there is lack information about the importance of children as a reservoir for infection of mosquitoes. This is a serious deficiency since, at least in Africa, children constitute a major part of the infectious reservoir.

5.3.2 No information could be collected on how long an individual remained infectious to mosquitoes. This made inaccurate the comparison in Figure 4.5 of Clinic data based on daily attendance rates and village data based on cross sectional surveys.

5.3.3 The production of large numbers of oocysts from individual donors who had no apparent gametocytes remains a paradox which was unexplained by (a) a consideration of the volume of blood taken up by mosquitoes and that observed on slides (b) a small study comparing blood meals with finger pricks.

5.3.4 In the present study we did not collect the number of mosquitoes which died before 7 days which was the day scheduled for dissection. Some of the deaths may have been due to heavy *P. falciparum* infection. Thus the infection rates reported may have been underestimates.

5.3.5 Several studies have shown that large mosquitoes tend to nurture large numbers of oocysts and mosquito size may affect the relationship between parasite prevalence and intensity of infection (Medley *et al.*, 1993). In this study, we did not measure the size of the mosquitoes, so heterogeneous mosquito size may have contributed to the variation within batches and to differences between batches.

5.3.6 Measurement error in the "independent" variable of regression analyses exploring the relationship between oocyst output and parasite (gametocytes or asexual stages) input was not taken into account, and this may also be partically resposible for the attenuation of regression slopes.

5.4 Recommendations for improvements in methods of malaria control

5.4.1 The results in chapter 3 showed that the parasite prevalence in the people who used treated nets was much lower than in those who did not used treated nets. However, only 44% of nets were treated. Therefore, the use of treated nets as well as their re-impregnation should be continuously promoted to cover all communities in the endemic areas, especially in people who stayed outside their villages in temporary huts for agricultural activities. Note should be taken of the national programme in Vietnam which provides free retreatment for the nets of 11 million people.

5.4.2 Follow up after treatment should be encouraged since the results showed that approximately 50% do not come back for a blood test after treatment. In an area notorious for multi-drug resistance this is dangerous both for the individual patient and for the spread of resistance in the population. In Mae Hong Son Province, some of patients are foreign nationals who are not permanently resident in Thailand so it is difficult to follow them up but more effort should be made.

5.4.3 Patients suffering from primary and first relapse attacks of *P. falciparum* malaria who are admitted to hospital for treatment usually stay for about 2 weeks and are then discharged (on clinical evidence) and return home. Normally, they are given only schizonticidal drugs and they are discharged just when they are becoming infective to mosquitoes. Thus the malaria staff should ensure that they are given gametocytocidal treatment.

5.4.4 In Mae Hong Son, there is a military camp. The soldiers patrol in the forest areas especially near the Thai-Myanmar border where they can acquire malaria easily. A gametocytocidal drug could be a most valuable weapon in preventing the spread of malaria among those troops and among people who regularly go into remote forest areas and could infect mosquito populations.

5.4.5 Active case detection by Mass Blood Survey (MBS) can be used to investigate malaria reservoirs in a community. The results from the present study showed that such surveys can identify most of people who can infect mosquitoes. Therefore, MBS should be expanded to cover all the populations who inhabit the endemic areas of Thailand, with the long term aim of eliminating the reservoirs of infection there. The results from the present study suggest that directing an anti-gametocyte drug (such as primaquine) to all feverish patients who are malaria infected in the villages could have a major impact on the reservoir of infection. First priority should be given to intensely endemic areas where the *Anopheles* density is high and gametocyte carriers are numerous. However, we have to bear in mind the side effects of primaquine, especially the risk of acute haemolysis in those with G6PD deficiency. Thus before giving this drug, tests for G6PD deficiency must be done.

5.5 Suggestions for further work

5.5.1 This work has dealt with only one small geographical location so more cross-sectional studies should be done in other parts of the country. Comparison of the prevalence of asymptomatic malaria in high and low transmission areas as well as their infectivity to other *Anopheles* species, should be made.

5.5.2 As pointed out in 5.3.1 the present study does not provide information on the infectiousness of children aged less than 15 years. No other studies on infectiousness of children age<15 years to mosquitoes have been reported in Thailand or elsewhere in south-east Asia. Feeding mosquitoes on children is not painful or dangerous to them and every effort should be made to persuade the Thai ethical committee that a study with children is important and justifiable, with due

regard to thorough explanation and careful gaining of consent from children and their parents.

5.5.3 More investigations on comparison of the number of gametocytes from finger pricks and mosquito blood meals are needed since the sample size in the present study was only six individuals. A finger prick often draws blood from deeper tissue than a mosquito proboscis can reach. Therefore, an additional question we need to ask is - are gametocytes circulating at the same density at different depths? The RT-PCR technique should be used to check for low densities of gametocytes in the paradoxical cases with no observable gametocytes by microscopy, but which yielded oocysts.

5.5.4 Primaquine is used widely in Thailand for *P. falciparum* in areas with low mefloquine resistance and for *P. vivax*, *P. ovale* and *P. malariae* infections. However, there are side effects of primaquine such as gastro-intestinal toxicity, and acute haemolysis especially in those with G6PD deficiency. Therefore, other anti-gametocyte drugs such as tafenoquine (WR 238605), pyronaridine or artesunate should be tested for their effects against the sexual stages. Studies of the infectivity of the gametocytes in mosquitoes after treatment with these drugs should be done using the methods described in chapter 4 of this thesis. The aim would be to develop a method of mass use of anti-gametocyte drugs to use as a form of transmission control to supplement vector control and, hopefully, a transmission blocking vaccine.

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APPENDICES

Annex 1. Raw data of Mass blood surveys and mosquito feeding

Annex 1.1 Slide positive in 30 villages from two cross-sectional surveys.

Village	Slide –ve	Slide +ve	Total
Doi Sang	223	0	223
Huai Karn	205	0	205
Huai Makur Som	559	0	559
Huai Pha	299	1	300
Huai Pong Kare	96	0	96
Huai Pong-on	330	4	334
Huai Pueng	32	1	33
Huai Pueng 2	265	3	268
Huai Salop	314	2	316
NAI Huai Sarn Nai	173	2	175
Huai Sarn Nork	129	2	131
Kung Mai Sak	240	0	240
Mae Sa Nga	295	1	296
Mae Suya	351	0	351
Mai Sa-pae	394	8	402
Mok Cham Paee	407	5	412
Na Pa Pak	216	0	216
Nai Soy	780	9	789
Namapin	165	0	165
Naplajard	255	8	263
Num Gud	301	0	301
Pakolo	210	0	210
Ruam Thai	102	0	102
Sop Pong	114	1	115

Annex 1.1 Continued

Village	Slide -ve	Slide +ve	Total
Sop Soy	230	4	234
Pang Moo	323	0	323
Suan Pa 40	33	1	34
Thong Muang	196	3	199
Thop Soak	284	2	286
Thung Masarn	234	0	234
Total	**7755**	**57**	**7812**

Annex 1.2 Data on dissection of mosquitoes fed on 28 malaria patients from village surveys.

Slide no.	Age(Years)	Species	Asexual densities /µl	Gametocyte densities/µl	No. of mosquitoes Dissected	No. of mosquitoes infected	No. oocysts	Geometric mean no. oocysts
157	20	Pf	1000	0	12	0	0	0
46	15	Pf	13280	0	19	0	0	0
57	17	Pf	60280	0	6	0	0	0
1	62	Pf	8400	0	10	0	0	0
30	15	Pf	200	0	12	0	0	0
87A	20	Pf	1360	0	17	0	0	0
35	15	Pf	840	0	25	0	0	0
61	23	Pf	13960	0	34	0	0	0
102	37	Pf	2560	0	23	0	0	0
37	29	Pf	55800	0	20	2	15	7.07
112	30	Pf	1600	80	36	7	470	40.54
21	38	Pf	1840	120	12	4	9	1.78
118	43	Pf	24520	1200	14	0	0	0
84	24	Pv	920	0	18	6	7	1.12
81A	33	Pv	2360	0	20	0	0	0

Pf= *P. falciparum*, Pv= *P. vivax*

Annex 1.2 Continued

Slide no.	Age(Years)	Species	Asexual densities /µl	Gametocyte densities/µl	No. of mosquitoes Dissected	No. of mosquitoes infected	No. oocysts	Geometric mean no. oocysts
97	22	Pv	200	0	7	0	0	0
78	37	Pv	2800	0	14	6	14	1.91
1A	31	Pv	40	0	18	0	0	0
87	15	Pv	2000	0	9	0	0	0
69	38	Pv	800	0	6	0	0	0
2	15	Pv	16120	1800	11	10	210	15.49
56	18	Pv	1760	200	35	8	73	7.75
36	70	Pv	1680	120	24	0	0	0
14A	28	Pv	18320	200	10	4	91	22.41
14	20	Pv	480	80	25	0	0	0
81	20	Pv	5920	160	17	7	40	5.46
74	16	Pv	7480	640	10	3	6	1.82
174	38	Pv	7040	3920	15	0	0	0

Pf= *P. falciparum*, Pv= *P. vivax*

Annex 1.3 Results of feeding to mosquitoes of 92 malaria patients from the Clinic.

Slide no.	Age (Years)	Species	Asexual densities /µl	Gametocyte densities/µl	No. of mosquitoes Dissected	No. of mosquitoes infected	No. oocysts	Geometric mean no. oocysts
MC44	45	Pf	5560	0	25	0	0	0
MC42	27	Pf	1400	0	7	0	0	0
MC61	44	Pf	1640	0	25	0	0	0
MC17	28	Pf	25000	0	25	5	238	12.09
MC21	47	Pf	2280	0	23	1	2	2.0
MC6	28	Pf	1440	0	10	0	0	0
MC99	18	Pf	8520	0	26	0	0	0
MC70	55	Pf	40960	0	19	0	0	0
MC10	34	Pf	1280	0	19	0	0	0
MC98	18	Pf	35240	0	5	0	0	0
MC62	27	Pf	280	0	32	0	0	0
MC18	18	Pf	36480	0	21	4	218	29.62
MC85	21	Pf	4160	0	6	0	0	0
MC38	22	Pf	3640	0	19	4	11	2.34
MC51	40	Pf	9040	0	13	0	0	0
MC83	39	Pf	680	0	7	0	0	0
MC100	15	Pf	2400	0	20	0	0	0
MC88	19	Pf	13160	0	5	0	0	0
MC96	41	Pf	920	0	13	0	0	0
MC13	17	Pf	1200	0	9	2	12	5.66
MC68	34	Pf	1800	0	8	0	0	0
MC57	52	Pf	12600	0	13	0	0	0
MC79	27	Pf	40560	0	7	0	0	0
MC14	18	Pf	24280	0	11	2	6	2.83
MC75	20	Pf	7240	0	6	1	3	3

Pf= *P. falciparum*, Pv= *P. vivax*

Annex 1.3 Continued

Slide no.	Age (Years)	Species	Asexual densities /µl	Gametocyte densities/µl	No. of mosquitoes dissected	No. of mosquitoes infected	No. oocysts	Geometric mean no. oocysts
MC66	15	Pf	27200	0	26	0	0	0
MC39	52	Pf	2000	0	16	0	0	0
MC2	36	Pf	720	0	15	0	0	0
MC58	22	Pf	31240	0	12	0	0	0
MC59	42	Pf	480	0	10	0	0	0
MC55	35	Pf	58000	0	12	0	0	0
MC65	17	Pf	800	0	13	0	0	0
MC76	29	Pf	24400	0	18	0	0	0
MC45	29	Pf	8000	0	22	0	0	0
MC91	63	Pf	72080	120	20	0	0	0
MC36	35	Pf	24680	80	16	2	6	2.83
MC50	45	Pf	1880	40	34	0	0	0
MC97	27	Pf	440	400	6	0	0	0
MC80	19	Pf	8840	40	5	0	0	0
MC28	20	Pf	320	160	7	1	8	8
MC33	21	Pf	4800	400	25	6	38	4.79
MC20	15	Pf	6000	160	23	5	43	4.16
MC5	15	Pf	360	40	5	0	0	0
MC9	21	Pf	7720	40	21	0	0	0
MC90	30	Pv	1600	0	6	0	0	0
MC22	51	Pv	1760	0	10	0	0	0
MC69	25	Pv	5240	0	14	0	0	0
MC19	25	Pv	20080	0	19	4	45	12.81
MC92	44	Pv	80	0	17	0	0	0
MC12	15	Pv	400	0	29	0	0	0
MC63	50	Pv	8920	0	13	0	0	0

Pf = *P. falciparum*, Pv = *P. vivax*

Annex 1.3 Continued

Slide no.	Age (Years)	Species	Asexual densities /µl	Gametocyte densities/µl	No. of mosquitoes dissected	No. of mosquitoes infected	No. oocysts	Geometric mean no. oocysts
MC11	21	Pv	16560	0	17	0	0	0
MC94	15	Pv	2000	0	12	0	0	0
MC95	43	Pv	1560	0	6	1	2	2
MC48	43	Pv	8280	0	25	24	2839	104.81
MC1	30	Pv	480	560	15	4	90	20.45
MC101	34	Pv	7160	600	26	12	28	1.95
MC16	37	Pv	3080	80	23	2	8	2.65
MC46	33	Pv	6160	320	26	26	1439	4.79
MC89	29	Pv	11240	1440	8	0	0	0
MC32	22	Pv	7120	120	12	2	20	7.14
MC41	38	Pv	1600	240	9	0	0	0
MC37	15	Pv	6520	800	19	13	71	4.22
MC52	28	Pv	2480	400	18	4	111	5.48
MC49	15	Pv	5840	800	25	9	18	1.79
MC78	45	Pv	6200	160	14	10	344	19.33
MC8	50	Pv	5880	1120	10	0	0	0
MC35	32	Pv	8280	1400	35	2	6	2.83
MC93	18	Pv	36720	8400	12	5	79	14.42
MC40	49	Pv	1880	1000	24	5	13	2.17
MC71	39	Pv	400	40	25	3	3	1.0
MC15	34	Pv	720	320	10	2	24	12.0
MC53	22	Pv	6000	800	15	0	0	0
MC27	17	Pv	15800	280	5	2	16	3.87

Pf = *P. falciparum*, Pv = *P. vivax*

Annex 1.3 Continued

Slide no.	Age (Years)	Species	Asexual densities /µl	Gametocyte densities/µl	No. of mosquitoes dissected	No. of mosquitoes infected	No. oocysts	Geometric mean no. oocysts
MC82	17	Pv	2600	440	5	0	0	0
MC72	39	Pv	4760	160	24	23	266	8.67
MC7	50	Pv	68640	1000	30	0	0	0
MC86	35	Pv	21760	480	9	0	0	0
MC56	37	Pv	8520	520	19	2	2	1.0
MC67	63	Pv	960	40	16	4	10	2.21
MC81	26	Pv	2120	320	6	0	0	0
MC54	18	Pv	2760	360	25	2	5	2.45
MC47	34	Pv	6080	40	25	24	462	13.65
MC4	17	Pv	19040	3360	29	0	0	0
MC84	17	Pv	1400	120	6	0	0	0
MC24	53	Pv	8600	80	7	1	2	2.0
MC64	23	Pv	3400	40	7	2	3	1.41
MC77	27	Pv	2520	120	5	1	4	4.0
MC34	60	Pv	6360	760	40	26	331	9.47
MC60	33	Pv	8960	240	16	10	73	5.75
MC87	30	Pv	1680	520	8	0	0	0
MC3	55	Pv	1640	840	8	2	46	12.96

Pf = *P. falciparum*, Pv = *P. vivax*

Annex 2. Questionnaire and blood smear form

Annex 2.1 Structured questionnaire

Slide number _____

Date _____

General data

1. ID No. _____	□□□□
2. Head of HH_____	
3. Name _____	
4. Sex _____	□
5. Age _____	□□
6. Tribe_____	□
7. Occupation _____	□
8. HH number _____	
9. Village _____	□□
10. Canton _____	□
11. Body temperature _____ ° C	□□□

Malaria history

12. Do you have a fever now?	□
___ Yes	
___ No	
13. Do you think you have had a fever in the previous week?	□
___ Yes_____ days	
___ No	
14. How many attacks of malaria do you think you have had in the last two years?	□□
_____ times	

15. When was the last time you had malaria? ☐

_____

16. Do you know what kind of malaria you have? ☐

___ *P. falciparum*

___ *P.vivax*

___ Mixed infection

17. Where did you go to have malaria test done? ☐

___ Health Centre

___ Hospital

___ Malaria Clinic

___ Others (specify)_____

18. Where did you get your treatment? ☐

___ Dispensary

___ Health Centre

___ Hospital

___ Malaria Clinic

5) Others (specify) _____

19. Did you return to the Clinic for a blood examination? ☐

___ Yes

___ No

Movement and mosquito protection

20. Have you been out of your village at night in the last four weeks? ☐

___ Yes

___ No

☐☐

21. If yes, how many nights did you spend away from the village?

_____ days

22. Did you do anything to protect yourself from mosquito biting? ☐

___ Yes

___ No

23. If yes, what kind of mosquito protection did you use? ☐

_____ mosquito coil _____ repellent

_____ spray _____ smoke

_____ mosquito net _____ others

(If you choose mosquito net, please continue item 24-27)

24. How many mosquito nets do you have in your family? ☐☐

_____ nets

25. Do you use mosquito net? ☐

___ Yes, every nights

___ Yes, sometimes

___ Do not use

26. How many people sleep per net? ☐

_____ persons

27. Have you had this mosquito net treated with insecticide? ☐

___ Yes

___ No

28. How many mosquito nets were treated? ☐☐

_____ nets

Name of interviewer _____

Date _____/_____/_____

Annex 2.2 Blood smear collection form

BLOOD RECORD FORM (EP.1)

☐ PCD (Malaria clinic, Hospital, Health centre, Village malaria volunteer, Village health volunteer)

☐ ACD, ☐ SCD, ☐ Follow up Form address: Group..... Village..... Canton ..... Amphur ..... Province.....

☐ Investigation, case no..... Area code.....

☐ Other (specified)..... Type of area ☐ A1 ☐ A2 ☐ B1 ☐ B2

☐ Code's name.....

(1)	(2)	Present address of patients							Age	Sex	(12)	Blood result						Drug treatment								Note
		(3)	(4)	(5)	(6)	(7)	(8)	(9)				Neg	F	V	M	Mix	Fg	C	SP	MSP	Q	T	(24)	(25)	(26)	

Date of receiving..... Receiver..... Blood examination date..... Examiner.....

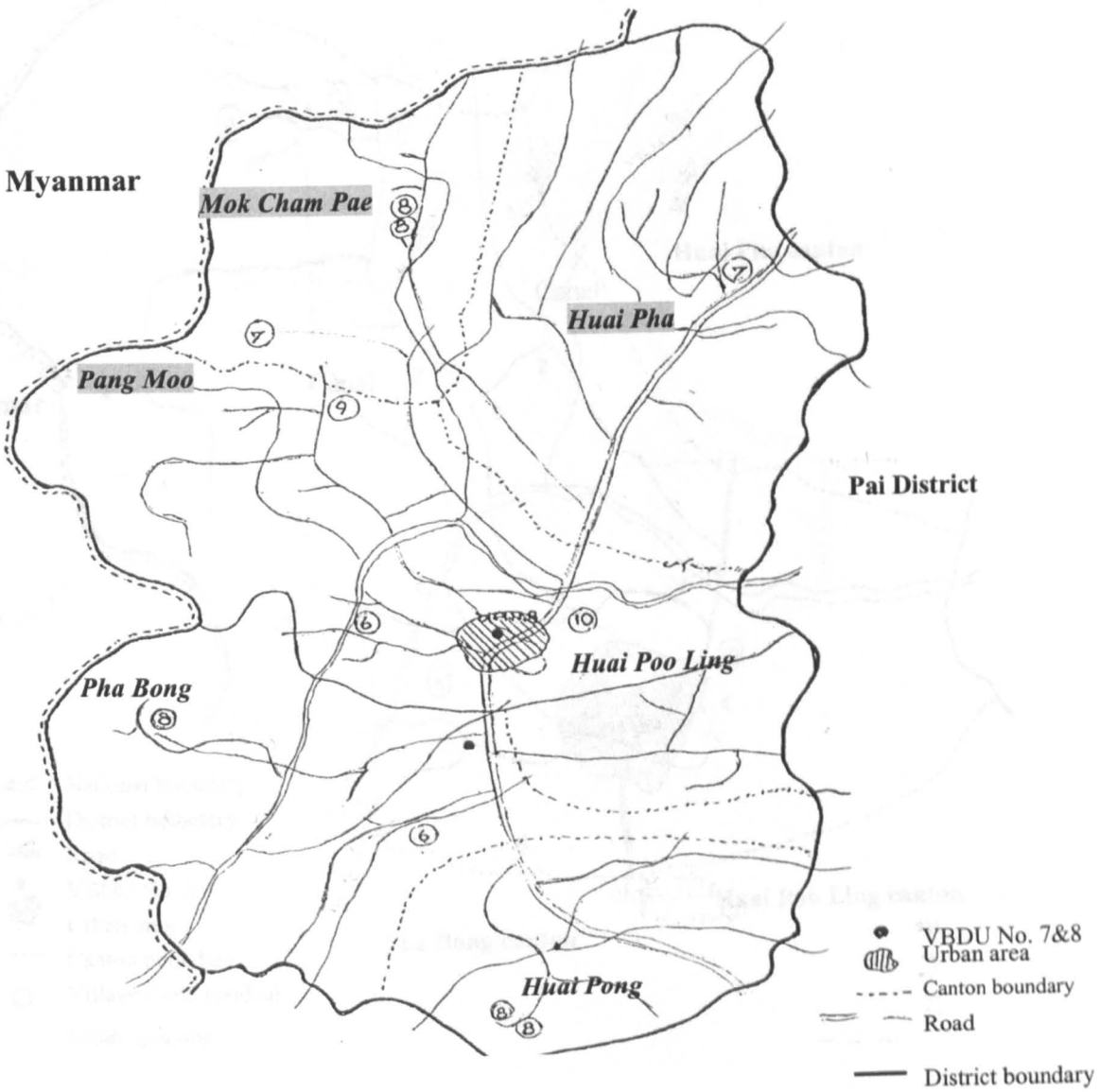
Note:

(1) =Blood smear date, (2) =Patient's name, (3) =Household owner's name, (4) =Household, (5) =Village group, (6) =Village no., (7) =Tambon, (8) = Amphur, (9) = Province, (12) = Film no., (24) = None, (25) = Primaquine (5mg), (26) = Primaquine (15mg)

Annex 3 Research team and maps of the study area

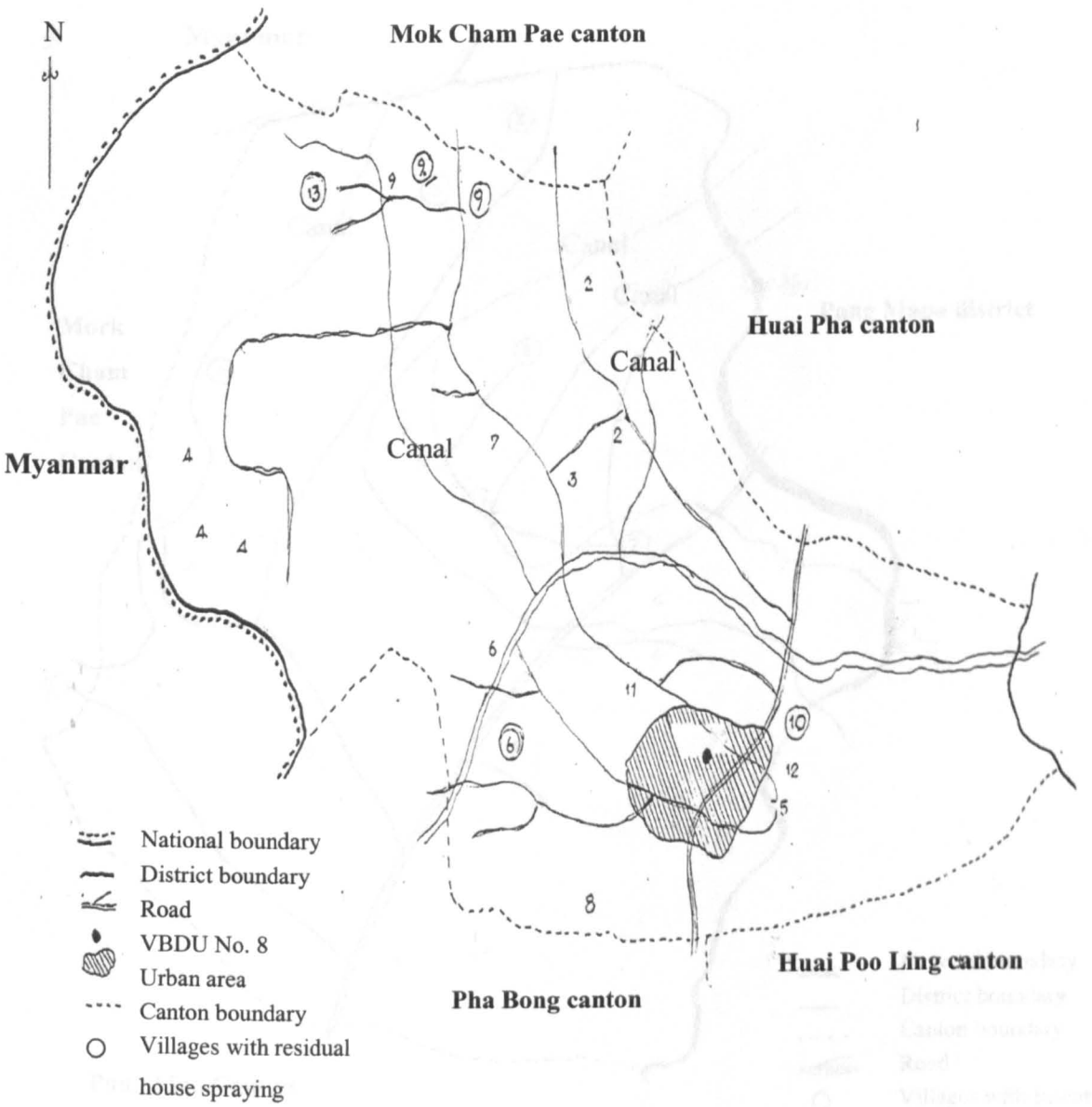
Annex 3.1 Research team and blood collection

Annex 3.2 Map of Muang District



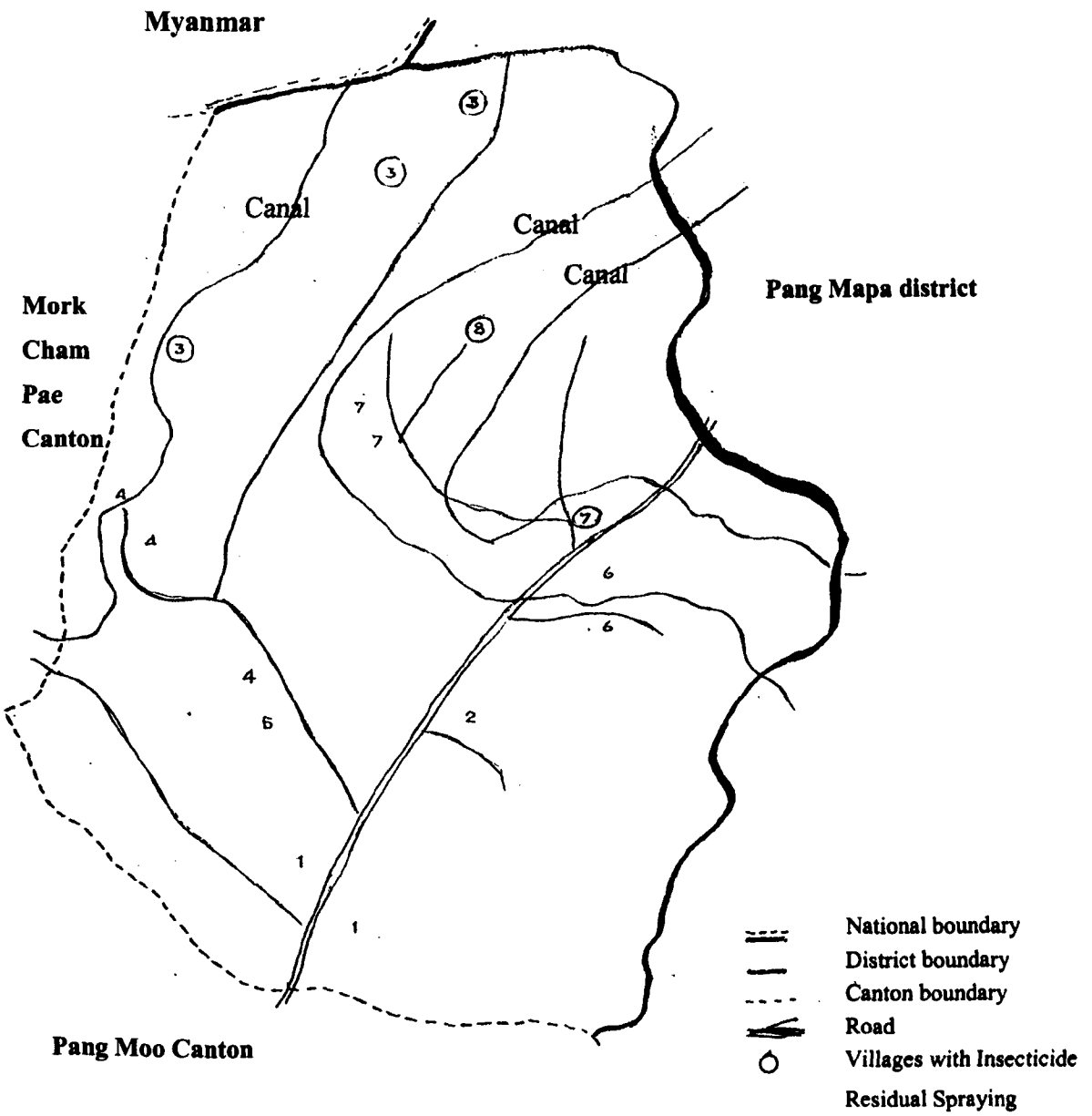
Annex 3.3 Map of Pang Moo canton

This canton contains 13 villages. The total population was 8,628 in 1999.



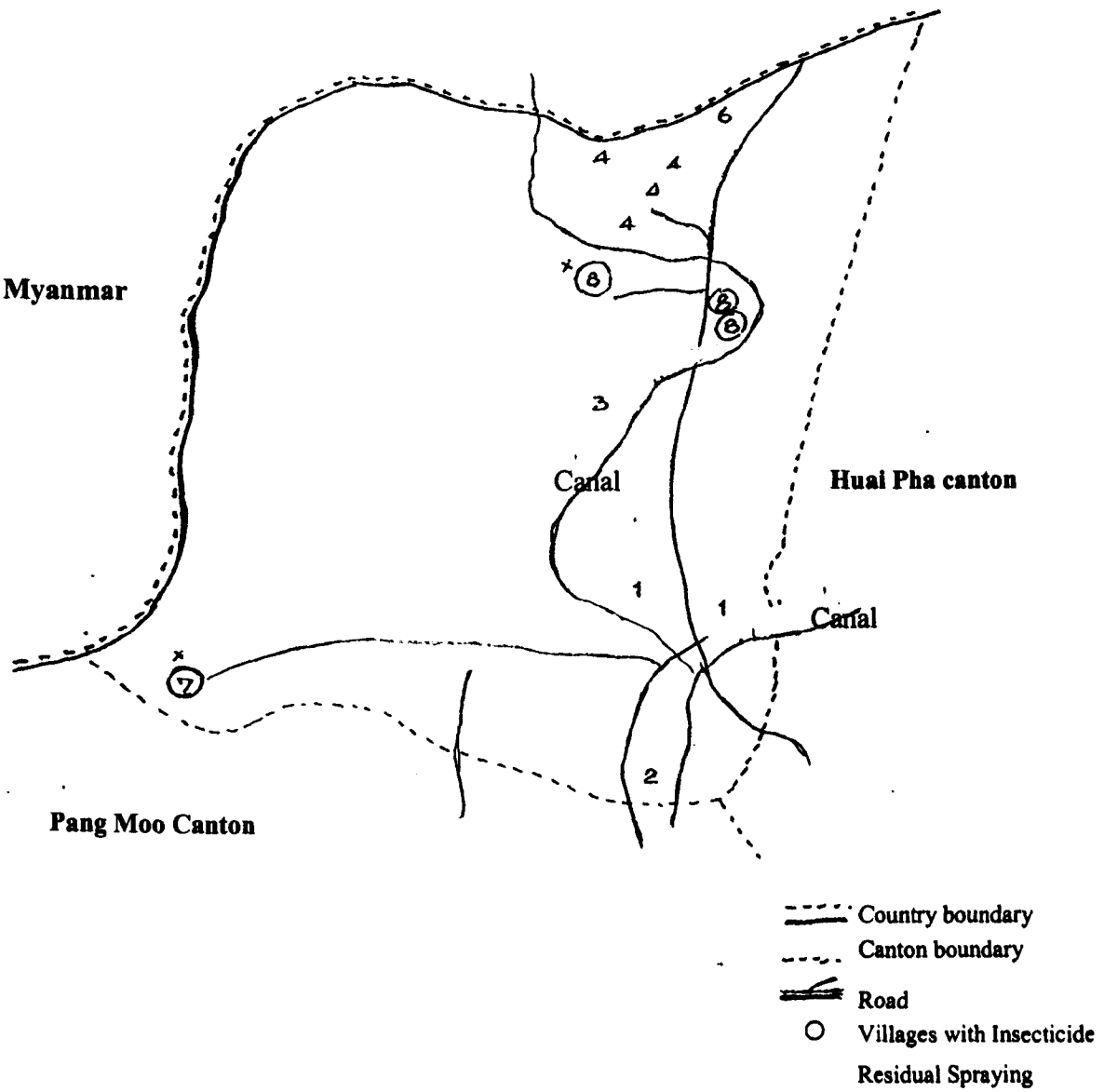
Annex 3.4 Map of Huai Pha canton

This canton has 8 villages. Population census in 2000 was 2,442.



Annex 3.5 Map of Mok Cham Pae canton

This canton consists 8 villages. The census population was 8,628 in 1999.



Annex 4 Ethical documents

Annex 4.1 Information sheet

Study title:	Importance of asymptomatic malaria and its infectivity to *Anopheles* mosquitoes in Mae Hong Son Province, Thailand
Researcher's name:	Miss Aree Pethleart
	Lecturer in Parasitology
Researcher's address:	Faculty of Medicine
	Thammasat university, Pathumtani, 12121 THAILAND
	Tel (66) (2) 926-9716, Fax (66) (2) 926-9675

This information sheet may have some facts you may not understand. Please feel free to ask the researcher and/or interviewer to explain anything you do not understand or would like more information on.

Introduction

This study is part of mass blood survey in Muang district of Mae Hong Son Province to learn about the asymptomatic malaria and its infectivity to *An. minimus* mosquitoes. Your responses will be used for malaria control programme to help reduce the malaria morbidity.

Objective of study

1. What is the epidemiology of asymptomatic malaria in healthy people in the study area?
2. What is the distribution of asexual and gametocyte parasite density in asymptomatic malaria?
3. Can the parasites from asymptomatic persons develop in *Anopheles* mosquitoes as well as those from symptomatic persons?
4. What is the percent positive and density of oocysts in mosquitoes fed on the blood of asymptomatic and symptomatic individuals.
5. How important is asymptomatic malaria in maintaining transmission

Study method

The proposed study is a combination of two designs. It consists of the field survey and a laboratory experiment. Mass blood survey is conducted twice a year. The people who have a positive slide will be interviewed. After signing consent forms, the 50 uninfected laboratory-bred *Anopheles* mosquitoes will be allowed to feed on each volunteer's arms.

Benefit from the study

A better understanding of the prevalence of asymptomatic an its infectivity to *Anopheles* mosquitoes will be used for the malaria control programme.

Incentive

You will not receive any money for participation in this research project

Human Right

You may refuse to participate or withdraw from this study at any time without any penalty or loss of services that you are entitled to. Your participation is completely voluntary and the result could be very helpful to other people in your community. Please do not sign your name in this form until you could have opportunity to ask any questions you want to and those questions concerning this study have been answered by researcher or interviewers.

Annex 4.2 Informed consent form

Study title:	Importance of asymptomatic malaria and its infectivity to Anopheles mosquitoes in Mae Hong Son Province, Thailand
Researcher's name:	Miss Aree Pethleart
	Lecturer in Parasitology
Researcher's address:	Faculty of Medicine
	Thammasat university, Pathumtani, 12121 THAILAND
	Tel: (66) (2) 926-9716, Fax: (66) (2) 926-9675

I have been informed that this study involves research which will be conducted by Miss Aree Pethleart, Faculty of Medicine, Thammasat University, a student at London School of Hygiene and Tropical Medicine, United Kingdom. I understand that this project is designed to study the importance of asymptomatic malaria and its infectivity to *Anopheles* mosquitoes in Mae Hong son province, Thailand. I understand that my participation in this study will involve the blood collection and direct feeding to *Anopheles* mosquitoes. I am aware that my involvement in this study will take approximately one hour of my time.

I understand that I may refuse to participate or withdraw from this study at any time without giving a reason and without affecting my normal care and management. I understand that my identity as a participant in this study will be kept in strict confidence and that no information that identifies me in any way will be released without my separate written approval. I am aware that all identifiable information will be protected to the limits allowed by law.

In this study, there are two points for asking consent:

1. Capillary blood collection

Capillary blood smears for quantitative parasite counts will be collected from each subject twice a year. I have been informed that the blood collection from finger prick will not cause serious problems for me. The wound will have cured itself within 7 days.

I have read this information sheet concerning this study and I understand what will be required of me if I take part of this study. I agree to take part in this study.

Participant's or Guardian's Signature	Date
Witness's Signature	Date

I cannot read but the researcher and her assistant have answered all of my questions concerning this study. I voluntarily agree to participate in this research project.

Participant's or Guardian's Signature	Date
Witness's Signature	Date

2. Direct feeding

In this study, 50 uninfected *An. minimus* mosquitoes will be fed on people who are slide positive. I have been informed that the direct feeding will not cause serious problems for me. The mosquito bites will have disappeared within 7 days. I have read this information sheet concerning this study and I understand what will be required of me if I take part of this study. I agree to take part in this study.

Participant's or Guardian's Signature	Date
Witness's Signature	Date

I cannot read but all of my questions concerning in this study have been answered by the researcher and her assistant. I voluntarily agree to participate in this research project.

Participant's or Guardian's Signature	Date
Witness's Signature	Date

Annex 4.3 Document from the Thai Ethical Committee, Ministry of Public Health

Ethical Review Committee
of
Research Committee, Ministry of Public Health

TITLE OF PROJECT	:	Importance of Asymptomatic Malaria and Its Infectivity to *Anopheles* mosquitoes in Mae Hong Son Province, Thailand
INVESTIGATOR	:	Miss Aree Pethleart
PLACE OF PROPOSED STUDY	:	1. Faculty of Medicine, Thammasat University 2. Faculty of Medicine, Chiang Mai University 3. London School of Hygiene and Tropical Medicine, University of London

APPROVED BY ETHICAL REVIEW OF RESEARCH COMMITTEE, MINISTRY OF PUBLIC HEALTH.

.....Chairman
(Director-General Department of Medical Services)

.....secretary
(Mr. Vichai Chokevivat)

DATE OF APPROVAL 18 AUGUST 1999

Annex 4.4 Document from the Ethical Committee of the London School of Hygiene & Tropical Medicine

LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE

ETHICS COMMITTEE

APPROVAL FORM
Application number: **603**

Name of Principal Investigator	**Aree Pethleart**
Department	**Infectious and Tropical Diseases**
Head of Department	**Professor Peter Smith**

Title **Importance of asymptomatic Malaria and its infectivity to *Anopheles* mosquitoes in Mae Hong Son Province**

Approval of this study is granted by the Committee.

Chair .....
(Professor Harrison Spencer, Dean)

Date 14 NOV 96

Comments from the Committee:

See attached

Any subsequent changes to the consent form must be re-submitted to the Committee.